Oxidative Medicine and Cellular Longevity

Transautophagy: Research and Translation of Autophagy Knowledge

Lead Guest Editor: Maria C. Albertini
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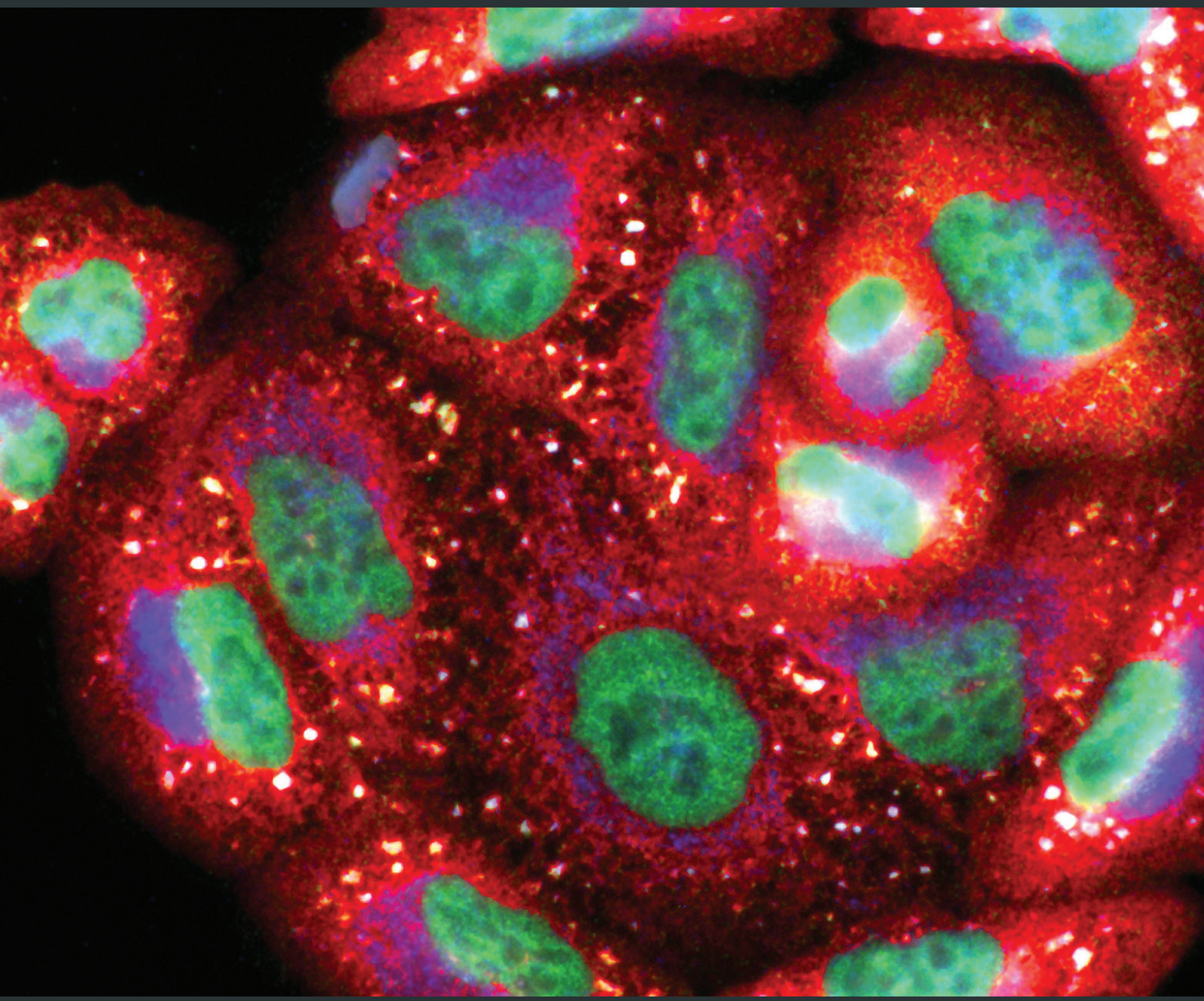

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Editorial Board

Contents

Hindawi
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Editorial

Transautophagy: Research and Translation of Autophagy Knowledge

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There is an urgent need to understand the process of autophagy in health and disease. Increasingly, human age-related diseases, such as cancer and neurodegeneration, as well as the great variety of metabolic disorders are found to correlate with significant alterations in both autophagy activity and capacity. However, implementing novel therapeutic treatment opportunities for combatting age-related human pathologies by targeting autophagy is unsatisfactorily slow. One of the reasons for this is that autophagy methods that can precisely monitor autophagy activity and capacity *in vivo* are currently nonexistent. Moreover, molecular details with regard to the complex regulation of autophagy in health and disease are still poorly understood. As a consequence, applying personalized medicine for targeting autophagy in patients suffering from age-related human diseases is still an unfulfilled aim.

In recognition of the requirement for developing next-generation autophagy knowledge and methodologies, the COST (European Cooperation in Science and Technology) Action (CA15138) "Transautophagy" (http://cost-transautophagy.eu/), a European network for multidisciplinary research on autophagy, has been successfully established by Caty Cases Louzao, Spain, and Patrice Codogno, France, in 2016. More than 200 autophagy researchers from over 28 European and neighboring countries aim to significantly advance autophagy research, concentrating on basic research on the autophagy molecular machinery (working group 1), strategies for autophagy analyses and modulation (working group 2), autophagy applications to crop and energy production (working group 3), biomedical research (working group 4), and biomedical translation and clinical trials (working group 5).

In this special issue, important further information on molecular mechanisms regulating autophagy is provided. These mechanisms highlight the relationship existing between cellular ROS levels and the process of autophagy. M. Pajares et al. reviewed publications related to the role of redox signaling in autophagy regulation with a special interest on ageing-associated changes and Alzheimer's disease. J. Jin et al. used primary acute myeloid leukemia (AML) patient samples and human AML cell lines to investigate the requirement for autophagy in AML differentiation. They suggested that granulocytic AML differentiation relies on noncanonical autophagy pathways and that restoring autophagic activity may be beneficial in therapies that aim at stimulating differentiation. N. Engedal et al. described novel aspects of oxidative stress-modulated miRNAs and elegantly demonstrated how an

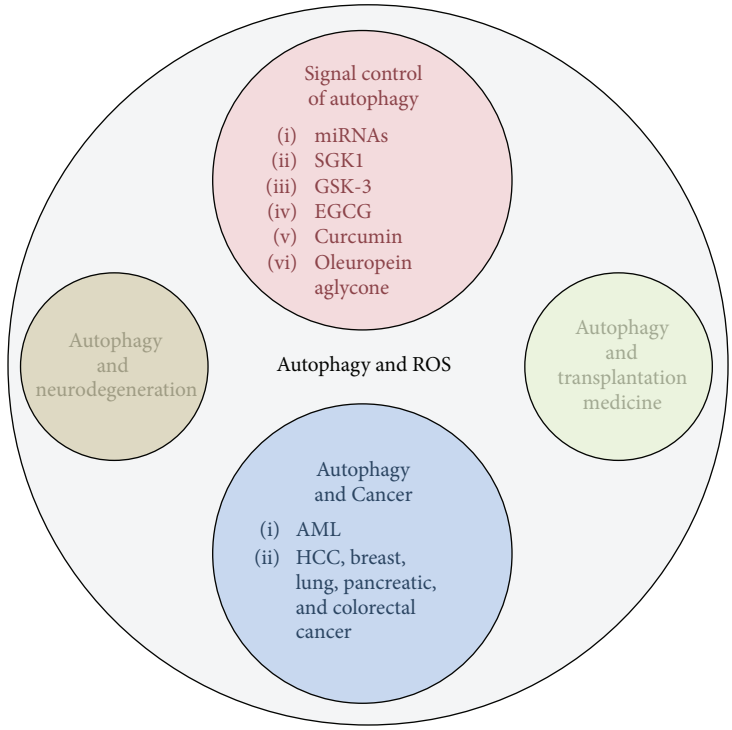

Figure 1: Summary of topics that either have been discussed in review articles or have been addressed in original studies in this special issue.

in silico approach can be employed to formulate hypotheses-driven research on the interrelation between miRNA-based gene regulation, oxidative stress signaling pathways, and autophagy. T. Zuleger et al. investigated the role of the serum- and glucocorticoid-induced protein kinase 1 (SGK1) in the control of autophagy. Their data strongly support the idea that SGK1 inhibits the process of autophagy. SGK1 seems to act upstream of ULK1 in regulating autophagy, and the authors provide a model whereby SGK1 regulates autophagy by contributing to the control of ULK1 gene expression. R. Mancinelli et al. reviewed the multifaceted roles of GSK-3 in cancer and autophagy-related diseases, in the context of the role of GSK-3 in several signaling pathways controlling a great variety of different key cellular functions.

Polyphenols, and other antioxidant molecules, have been considered as compounds that modulate the process of autophagy. M. Holczer et al. investigated the role of epigallocatechin-3-gallate (EGCG), the major polyphenol of green tea, in promoting autophagy-dependent survival. Their findings, obtained using HEK293T cells *in vitro*, revealed that EGCG treatment induced cytoprotective autophagy and increased cell viability by downregulating mTOR and upregulating AMPK signaling. S. Hasanbašić et al. showed that a set of polyphenolic antioxidants, in particular curcumin, can combat the process of protein aggregation *in vitro*. However, some antioxidants, such as vitamin C and NAC may have opposing effects at higher concentrations. The authors suggest that the level of protein aggregation may act as a sensor in order to ultimately prevent further cellular damage. C. Miceli et al. explored the effects of OA (Oleuropein aglycone, the main polyphenol found in olive oil) in cardiomyocytes by overexpression of monoamine oxidase-A (MAO-A). The authors observed that OA treatment counteracted the cytotoxic effects of MAO-A by restoring the MAO-A-induced defect in autophagic flux, most probably via activation and nuclear translocation of TFEB (transcription factor EB).

Finally, further studies focused on the role of oxidative stress and autophagy in different pathologies and new therapeutic strategies. M. Marinkovic et al. summarized the current knowledge of the interplay between autophagy regulation and five of the most life-threatening and prevalent malignancies (pancreatic, breast, hepatocellular, colorectal, and lung cancer). In addition, the authors present an overview of the recent advances in therapeutic strategies involving autophagy modulators in cancer therapy. A. Van Erp et al. reviewed knowledge on the crosstalk regulation between oxidative stress and autophagy in the context of transplantation medicine. C. Fang et al. discussed the implication of ROS and autophagy in both the onset and development of neurological disorders. The authors highlighted the interplay between ROS and autophagy in establishing a determinant role in the modulation of neuronal homeostasis, by an as yet unexplored mechanism, important in cerebral ischemia, AD (Alzheimer's disease), and PD (Parkinson's disease).

In summary, this special issue provides several new aspects in autophagy research with emphasis on translation of knowledge to applications, critically including the topic of autophagy regulation upon oxidative stress, aging, age-related diseases, and beyond (Figure 1).

Acknowledgments

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Research Article

SGK1 Inhibits Autophagy in Murine Muscle Tissue

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Background/Aims. As autophagy is linked to several pathological conditions, like cancer and neurodegenerative diseases, it is crucial to understand its regulatory signaling network. In this study, we investigated the role of the serum- and glucocorticoid-induced protein kinase 1 (SGK1) in the control of autophagy. *Methods.* To measure autophagic activity *in vivo*, we quantified the abundance of the autophagy conjugates LC3-PE (phosphatidylethanolamine) and ATG12-ATG5 in tissue extracts of SGK1 wild-type ($Sgk1^{+/+}$) and knockout ($Sgk1^{-/-}$) mice that were either fed or starved for 24 h prior sacrifice. *In vitro,* we targeted SGK1 by RNAi using GFP-WIPI1 expressing U-2 OS cells to quantify the numbers of cells displaying newly formed autophagosomes. In parallel, these cells were also assessed with regard to LC3 and ULK1 by quantitative Western blotting. *Results.* The abundance of both LC3-PE (LC3-II) and ATG12-ATG5 was significantly increased in red muscle tissues of SGK1 knockout mice. This was found in particular in fed conditions, suggesting that SGK1 may keep basal autophagy under control in red muscle *in vivo*. Under starved conditions, significant differences were observed in SGK1-deficient white muscle tissue and, under fed conditions, also in the liver. *In vitro*, we found that SGK1 silencing provoked a significant increase of cells displaying WIPI1-positive autophagosomes and autophagosomal LC3 (LC3-II). Moreover, autophagic flux assessments revealed that autophagic degradation significantly increased in the absence of SGK1, strongly suggesting that SGK1 inhibits both autophagosome formation and autophagic degradation *in vitro*. In addition, more ULK1 protein lacking the inhibitory, TORC1-specific phosphorylation at serine 758 was detected in the absence of SGK1. *Conclusions.* Combined, our data strongly support the idea that SGK1 inhibits the process of autophagy. Mechanistically, our data suggest that SGK1 should act upstream of ULK1 in regulating autophagy, and we hypothesize that SGK1 contributes to the regulation of ULK1 gene expression.

1. Introduction

Macroautophagy (referred to as autophagy) is a catabolic pathway that degrades cytosolic components, like proteins and damaged organelles. The cargo is engulfed by newly formed double-membrane vesicles, termed autophagosomes, which fuse with lysosomes for cargo degradation. The degraded monomers are released to the cytoplasm for recycling processes engaging anabolic pathways. Dysregulation of autophagy often results in pathological conditions like cancer and metabolic and neurodegenerative diseases [1, 2].

Autophagosome formation is tightly regulated by ATG (autophagy-related) proteins [2]. Amongst the ATG proteins, the ULK1 protein is crucial for the initiation of autophagosome formation and interacts with both the AMP-activated protein kinase (AMPK) and the mechanistic target of rapamycin complex 1 (mTORC1), the energy and nutrient sensors of the eukaryotic cell. Under low energy levels, AMPK activates, and under nutrient-rich conditions, mTORC1 inhibits ULK1 and autophagy [3, 4]. The generation of phosphatidylinositol 3-phosphate (PI3P) by the phosphatidylinositol 3-kinase class III (PI3KC3) is essential

for the nucleation of autophagosome formation and occurs downstream of ULK1 [5, 6]. PI3P recruits the WD-repeat protein interacting with phosphoinositide (WIPI) proteins (WIPI1–4) to the membrane origin for autophagosome formation [7]. Upon autophagy induction, the WIPI proteins are recruited to autophagosomal membranes; visualizing their localization has been established to assess autophagic activity by fluorescence microscopy (WIPI puncta formation) [8, 9]. Conjugation of LC3 to phosphatidylethanolamine (PE) by the ATG12 and LC3 ubiquitin-like conjugation systems is necessary for the elongation and closure of the autophagosomal membrane [2]. Lipidation can be measured by distinguishing the nonlipidated, cytosolic form of LC3 (LC3-I) from the lipidated, membrane-bound form of LC3 (LC3-PE or LC-II) by Western blotting. Increased levels of the ATG12-ATG5 conjugate and lipidated LC3 suggest increased autophagy [10, 11].

The serine/threonine-specific kinase SGK1 [12] was identified as a serum- and glucocorticoid-responsive gene in rat mammary epithelial tumour cells [13] and as a cell volume-sensitive transcript in a human hepatocellular carcinoma cell line [14]. SGK1 expression and activity can be induced via various stimuli like hormones, cytokines, and cellular stress [15]. SGK1 is regulated by the PI3K pathway, serine 422 is phosphorylated by mTORC2 [16, 17], which facilitates the phosphorylation of threonine 265 by PDK1 [15, 18]. Among others, SGK1 is involved in osmoregulation, transcription factor regulation, and cell proliferation [15, 19, 20]. Despite its broad function, SGK1-deficient mice develop a mild phenotype, which only becomes apparent under a challenging condition [21].

As both mTORC1 [22] and mTORC2 [16, 17] activate SGK1 and inhibit autophagy, SGK1 is considered an inhibitor of autophagy. H_2S was shown to suppress autophagy via stimulation of SGK1 [23], and inhibition of SGK1 induces LC3 lipidation and BECN1 (member of the PI3KC3 complex) expression in human glioblastoma cells [24]. SGK1 also inhibits FOXO3A [25–27], a transcription factor of autophagy genes [28]. In spite of the previous data, the mechanism of autophagy regulation by SGK1 is not understood. Here we show that red muscle tissue in SGK1 knockout mice has increased LC3-II and ATG12-ATG5 levels, suggesting increased autophagic activity. *In vitro*, we observed that SGK1 silencing provoked a significant increase of the autophagic flux as analyzed by GFP-WIPI1 puncta formation and LC3 lipidation. In addition, we found that the abundance of ULK1 protein lacking the inhibitory TORC1-dependent phosphorylation site increased in the absence of SGK1. Based on our findings, we entertain the hypothesis that SGK1 may control autophagy through ULK1.

2. Methods

2.1. Sgk−/− Mice. All animal experiments were approved by local authorities, adhered to the German law for the welfare of animals, and conducted in the Florian Lang laboratory. Mice deficient in SGK1 (*Sgk1*−/−) were bred and genotyped as previously described [21]. The experiments were conducted in mice on an original SV129 background [21]. SGK1 knockout (*Sgk1*−/−) and SV129 wild-type control mice (*Sgk1*+/+) of identical age (3 months) were fed *ad libitum* or starved for 24 h by keeping free access to tap drinking water. For subsequent tissue dissections, mice were anaesthetized with isoflurane and sacrificed by cervical dislocation. Organs were immediately harvested and flash frozen in liquid nitrogen.

2.2. Protein Extracts and Western Blotting Using Mouse Tissues. Tissue samples from the liver, kidney, heart, white muscle, red muscle, and aorta were collected from SGK1 knockout mice (*Sgk1*−/−, $n = 6$; 2 females, 4 males) and wild-type control (*Sgk1*+/+, $n = 6$; 2 females, 4 males) mice [21] that were either fed ($n = 3$) or starved ($n = 3$). Depending on the weight, samples were mixed with 1–1.5 ml RIPA buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0, 0.1% SDS, 1% deoxycholic acid, 1% NP-40) supplemented with protease inhibitor (Roche, 04693159001) and sonicated four times at 30.000/min for 15 seconds with a PT-DA 2105/2EC rotor (Polytron) in order to extract the proteins. After sonication, the samples were centrifuged at 14.000 rpm for 20 minutes at 4°C. 500 μl of the supernatant was mixed with 500 μl 2x Laemmli buffer (50 mM Tris pH 6.8, 1.25 mM EDTA pH 8.0, 12.5% glycerine, 2% SDS, 50 mM DTT, 2.5% β-mercaptoethanol, 0.025% bromophenol blue) and boiled for 5 minutes. The proteins were separated by SDS-PAGE and blotted on PVDF membranes (Millipore, IPVH00010). For LC3 detection 15% and for ATG12 detection 10%, SDS-PAGE gels were prepared. The following antibodies were used: LC3 (NanoTools, 0231-100/LC3-5F10), ATG12 (Abgent, ASC10625), α-tubulin (Sigma-Aldrich, T6074), anti-mouse IgG-HRP (Cell Signaling, 7076), and anti-rabbit IgG-HRP (Cell Signaling, 7074). LC3-positive control was purchased from NanoTools. ECL detection was performed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0100). Images were taken using the Fusion SL Vilber Lourmat device. The FUSION-CAPT Advance Software (Vilber Lourmat) was used for quantification.

2.3. Cell Culture. U-2 OS cells stably expressing GFP-WIPI1 were cultured in DMEM (Life Technologies, 31966) supplemented with 10% FCS (PAA, A15–101), 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies, 15140), 0.6 mg/ml G418 (Life Technologies, 11811098) at 37°C, and 5% CO_2.

2.4. Transient Transfections. Transient knockdown experiments with siRNA (SGK1 siRNA (h), Santa Cruz, sc-38913; control siRNA-A, Santa Cruz, sc-37007) were conducted by using Lipofectamine RNAiMAX (Invitrogen; 13778-075) according to the manufacturer's reverse transfection protocol. Briefly, in each well of a 24-well plate, 50 nM siRNA and 1 μl Lipofectamine RNAiMAX were diluted in 96 μl OPTI.MEM (Life Technologies; 51985-026) and the transfection solution incubated for 20 min at room temperature. Finally, 40,000 (for WIPI1 puncta

formation analysis) or 50,000 (for Western blotting) cells (U-2 OS GFP-WIPI1) in 500 μl DMEM/10% FCS were added to the transfection solution for 63 h. Knockdown events were verified by immunoblotting.

2.5. WIPI1 Puncta Formation Analysis. After transient transfection, 3 h treatments were performed under fed (DMEM/10% FCS) or serum and amino acid starvation (EBSS, Earl's balanced salt solution, Sigma-Aldrich, E2888) conditions in the presence and absence of 200 nM bafilomycin A1 (AppliChem, A7823). The cells were fixed with 3.7% PFA and mounted on slides using ProLong (Life Technologies, P36930). Up to 1541 cells (per treatment) from 3 independent experiments were counted manually by fluorescence microscopy (Zeiss, Axiovert 200M), and the percentages of cells positive for GFP-WIPI1 puncta were calculated. Representative images were taken with an Axiocam MRm camera using a 40x objective.

2.6. Protein Extracts and Western Blotting Using U-2 OS Cells. GFP-WIPI1 expressing U-2 OS cells were treated as described above for WIPI1 puncta formation analysis, except that after the 3 h treatment period, cells were lysed with hot 2x Laemmli buffer (50 mM Tris pH 6.8, 1.25 mM EDTA pH 8.0, 12.5% glycerine, 2% SDS, 50 mM DTT, 2.5% β-mercaptoethanol, 0.025% bromophenol blue), scraped into Eppendorf tubes, and sheered with a 23G injection needle. 40 μl of the extracts was loaded on a 10% or 15% SDS-PAGE gel, blotted on to a PVDF membrane (Millipore, IPVH00010), and incubated with the following antibodies: SGK1 (Cell Signaling, 12103), LC3 (NanoTools, 0231–100/LC3-5F10), phospho-ULK1 (S757) (Cell Signaling, 6888), ULK1 (Cell Signaling, 8054), α-tubulin (Sigma-Alderich, T6074), anti-mouse IgG-HRP (Cell signaling, 7076), and anti-rabbit IgG-HRP (Cell Signaling, 7074). Subsequently, ECL detection was performed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34096). Images were taken using the Fusion SL Vilber Lourmat device.

2.7. Statistics. Western blot and fluorescence microscopy results were analysed using SAS JMP 11.1® and two-tailed heteroscedastic t-testing. Statistical analyses with p values can be found in the supplementary data set (Suppl. Data Set (available here)).

3. Results

3.1. Autophagy Is Increased in SGK1-Deficient Mice. To investigate the effect of SGK1 in autophagy, we extracted proteins from liver, kidney, heart, aorta, red and white muscle of SGK1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice ($n = 6$) that were either fed ($n = 3$) or starved (24 h, $n = 3$) prior to cervical dislocation and tissue dissection. Subsequently, we analysed the abundance of the autophagy marker LC3-II (Figures 1–4) and ATG12-ATG5 (Figures 1 and 4) by quantitative Western blotting.

We found that the abundances of both LC3-II (Figure 1(a), upper panel and lower left panel) and ATG12-ATG5 (Figure 1(b), upper panel and lower left panel) were significantly increased in red muscle tissue derived from mice deficient for SGK1 (*Sgk1*−/−). In particular, this increase was found when fed mice were only compared with regard to LC3-II abundances (Figure 1(a), lower right panel: *Sgk1*+/+ versus *Sgk1*−/−, fed). Of note, red muscle tissue was the only organ with which we detected a significant difference between fed and starved conditions in the SGK1 wild-type background with regard to LC3-II (Figure 1(a), lower right panel: *Sgk1*+/+, fed versus starved). Based on this, we provide comparisons of SGK1 wild-type and deficient mice irrespective of their nutritional status (Figures 1–4, lower left panels) or with regard to fed and starved conditions (Figure 1–4, lower right panels). Raw data and statistical analyses for Figures 1–4 are provided (Suppl. Data Set).

In the white muscle tissue (Figure 2(a)) and liver (Figure 2(b)), we observed only a weak difference between *Sgk1*+/+ and *Sgk1*−/− mice, apparently when starved conditions were compared in white muscle tissue (Figure 2(a), lower right panel: *Sgk1*+/+ versus *Sgk1*−/−, starved) or fed conditions in liver (Figure 2(b), lower right panel: *Sgk1*+/+ versus *Sgk1*−/−, fed). Furthermore, in the heart (Figure 3(a)), an increase in LC3-II was observed (Figure 3(a), upper panel), but this increase was not significant (Figure 3(a), lower left panel: p value 0.06769). In the aorta, an increase of nonconjugated LC3 (LC3-I) was apparent (Figure 3(b), upper panel) but this had no influence on the abundance of LC3-II, which was not significant when *Sgk1*+/+ and *Sgk1*−/− mice were compared (Figure 3(b), lower panels). In the kidney tissue (Figure 4), we observed a slight increase in LC3-II; however, this was found not to be significant (Figure 4(a), lower panels), consistent with no significant alterations with regard to ATG12-ATG5 (Figure 4(b)).

In summary, our tissue screening approach assigned an inhibitory role of SGK1 on autophagy in the red muscle tissue.

3.2. SGK1 Inhibits Autophagy In Vitro. To investigate the role of SGK1 *in vitro*, we used a U-2 OS cell model established for the specific detection of autophagosomal membranes (puncta) decorated with the WIPI1 PI3P effector in autophagy [9]. Of note, due to its low endomembrane content, the U-2 OS cell line is superior for the fluorescence-based detection of newly formed membranes, such as autophagosomes. Here, we transiently transfected U-2 OS cells stably expressing GFP-WIPI1 with siRNAs targeting endogenous SGK1 (siSGK1) [29], along with nontargeting control siRNAs (siControl) (Figures 5–7). To adhere to the standard conditions that modulate autophagy, we used fed conditions (Fed), as well as starvation conditions (Starved) where we treated the cells with EBSS for maximal induction of autophagy *in vitro* [8], and to distinguish between an induction or a block of autophagy, we applied the lysosomal inhibitor bafilomycin A1 [10, 30] (Figures 5–7).

As expected, starvation conditions elevated the number of cells displaying autophagosomal membranes with GFP-WIPI1 that further increased when lysosomes were inhibited (+BafA1) (Figure 5(a), 5(b)). Further, we observed that upon SGK1 silencing (siSGK1) GFP-WIPI1 puncta prominently increased (Figure 5(a); representative images from starved cells are shown). This observation became even more

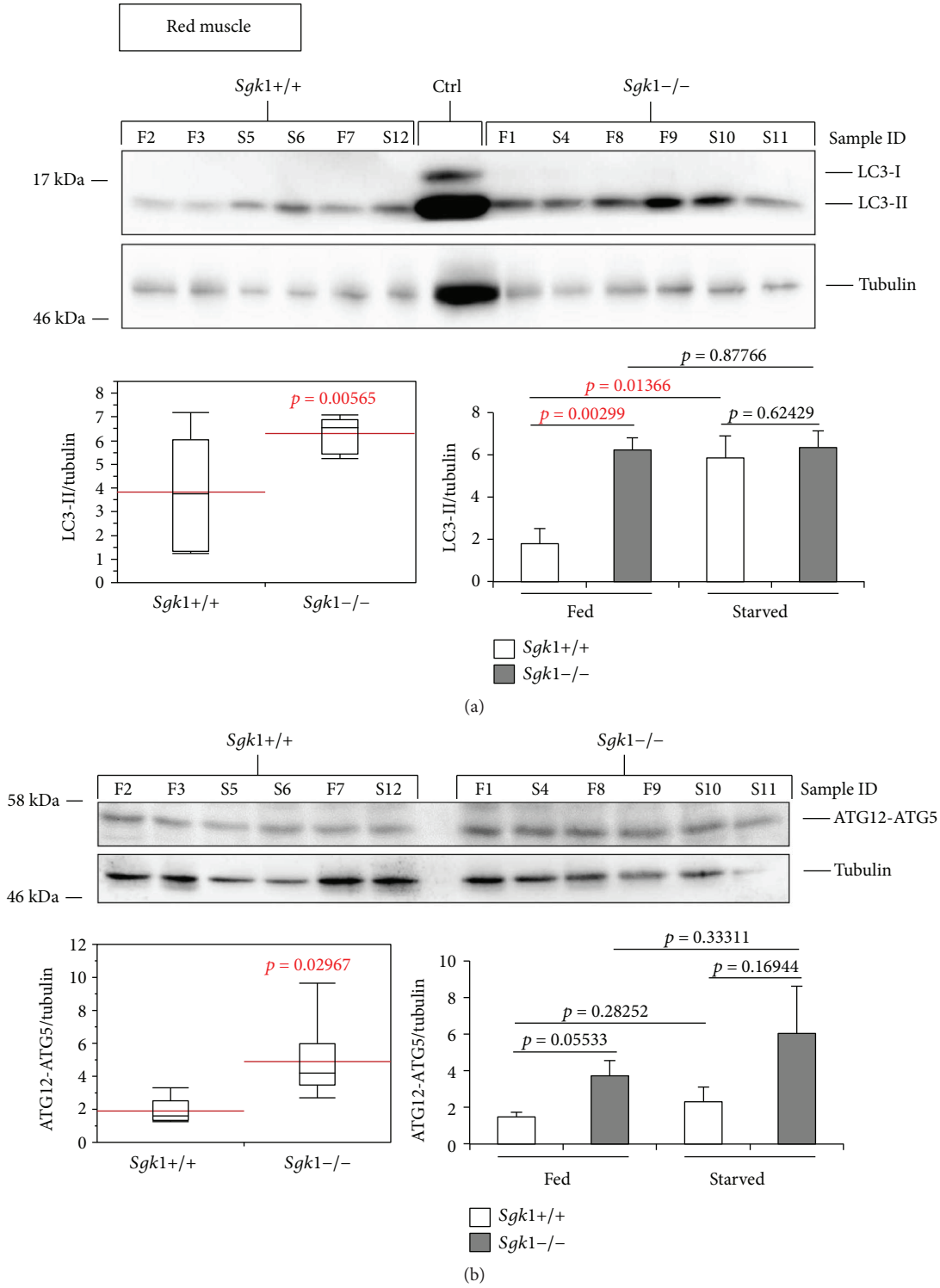

Figure 1: LC3-II and ATG12-ATG5 abundances are significantly increased in the red muscle tissue of Sgk1 knockout mice. LC3 lipidation was analysed by Western blotting (upper panel) and quantified (lower panels) in protein extracts from the red muscle of Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice ($n = 6$) (a). Of those, mice were either fed (F, $n = 3$) or starved (S, $n = 3$) as indicated (sample ID). LC3-positive control (Ctrl) was used to indicate the migration of nonlipidated LC3-I and lipidated LC3-II. Red lines on the box plots (lower panel, left) represent the mean values of all 6 Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice (lower panel, left) or with regard to fed and starved conditions (lower panel, right). p values are provided. In parallel, the abundance of the ATG12–ATG5 (ATG12–5) conjugate was likewise assessed (b). Supporting data is provided (Suppl. Data Set).

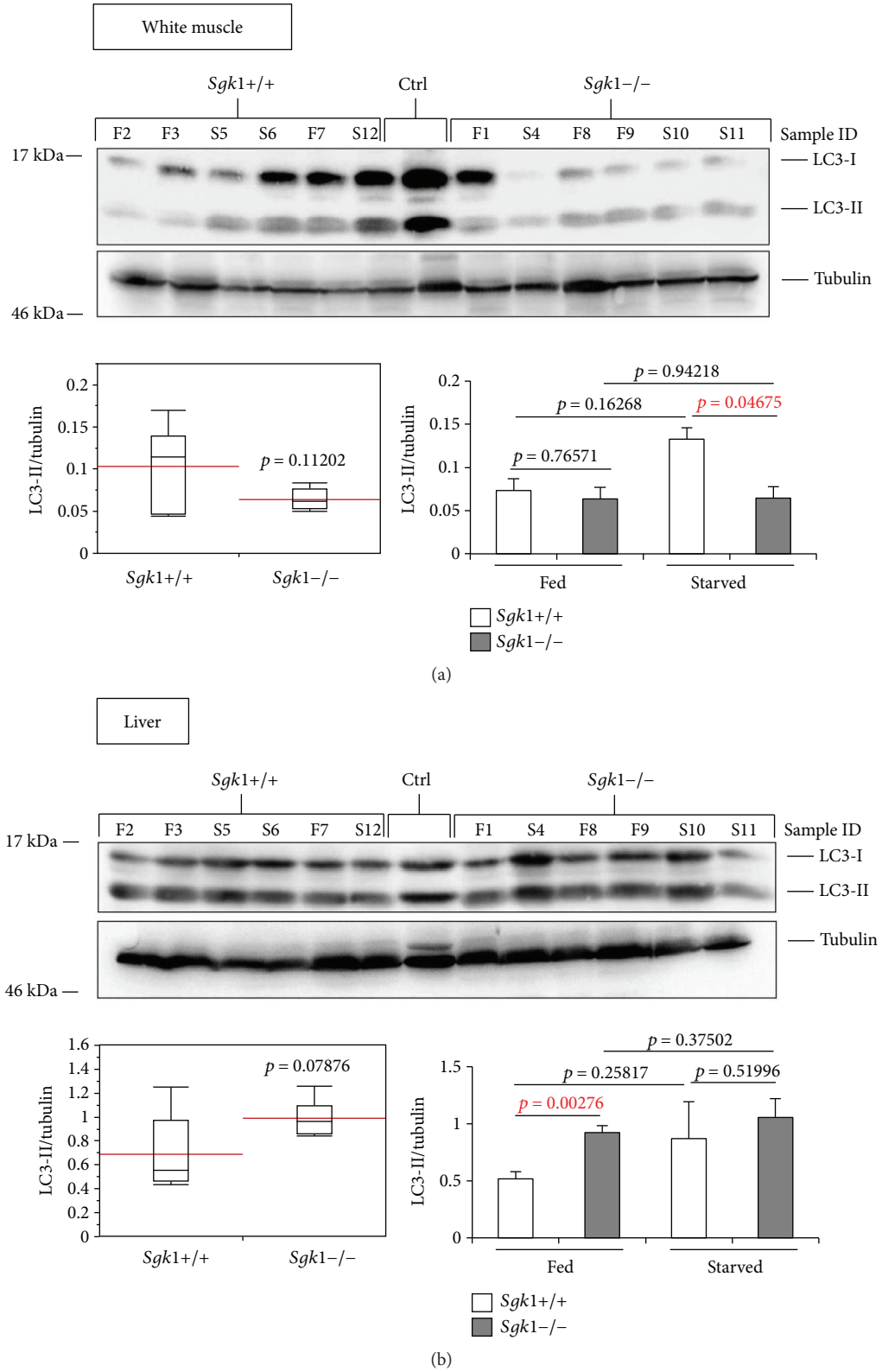

Figure 2: LC3-II abundance appears weakly altered in the white muscle and liver tissue of Sgk1 knockout mice. LC3 lipidation was analysed by Western blotting (upper panel) and quantified (lower panels) in protein extracts from the white muscle (a) or liver (b) of Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice ($n = 6$). Of those, 3 mice were either fed (F) or starved (S) as indicated (sample ID). LC3-positive control (Ctrl) was used to indicate the migration of nonlipidated LC3-I and lipidated LC3-II. Red lines on the box plots (lower panel, left) represent the mean values of all 6 Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice (lower panel, left) or with regard to fed and starved conditions (lower panel, right). *p* values are provided. Supporting data is provided (Suppl. Data Set).

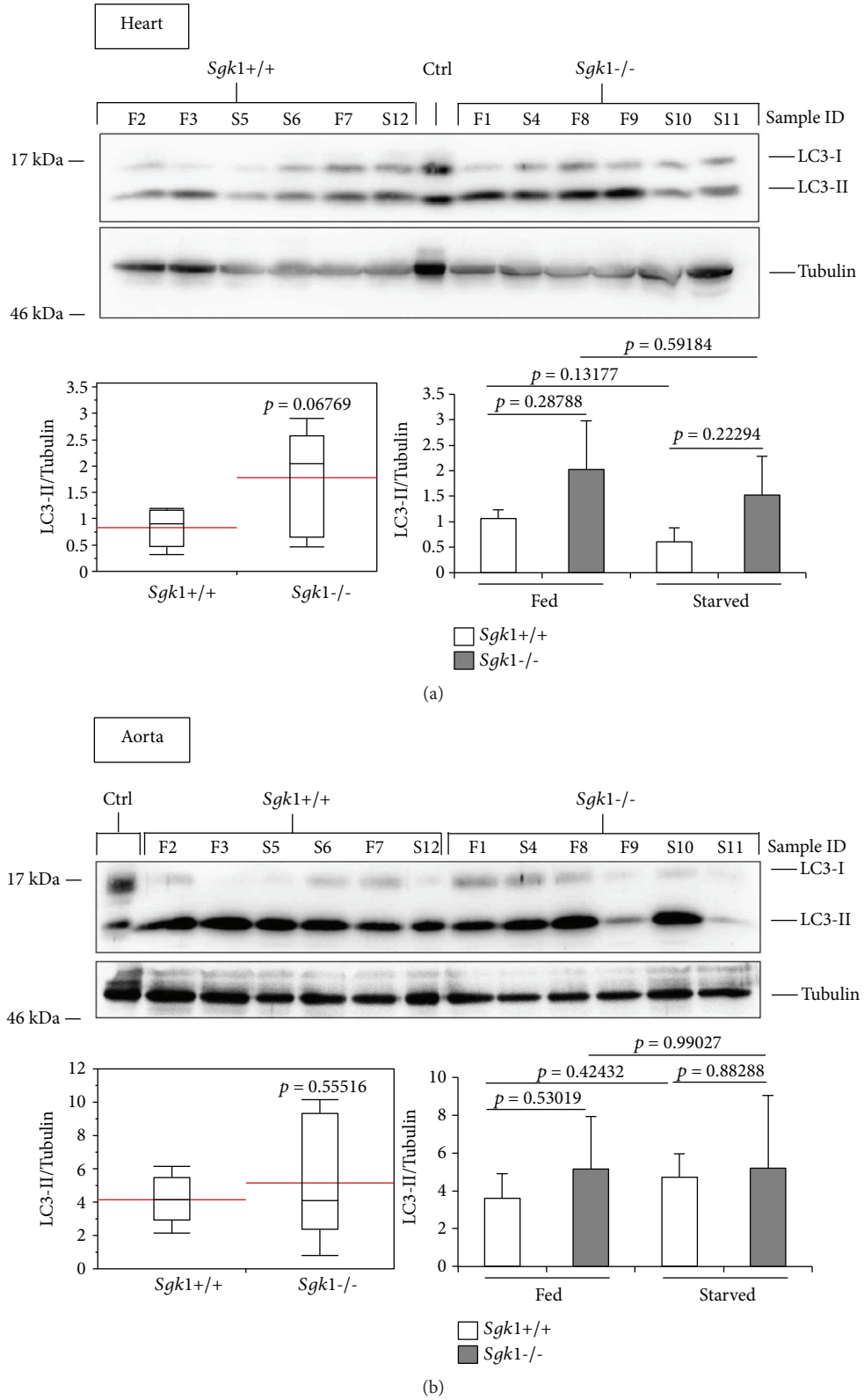

Figure 3: LC3 lipidation is unaltered in the heart and aorta tissue derived from Sgk1 knockout mice. LC3 lipidation was analysed by Western blotting (upper panel) and quantified (lower panels) in protein extracts from the heart (a) or aorta (b) of 6 Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice. Of those, 3 mice were either fed (F) or starved (S) as indicated (sample ID). LC3-positive control (Ctrl) was used to indicate the migration of nonlipidated LC3-I and lipidated LC3-II. Red lines on the box plots (lower panel, left) represent the mean values of all 6 Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice (lower panel, left) or with regard to fed and starved conditions (lower panel, right). *p* values are provided. Supporting data is provided (Suppl. Data Set).

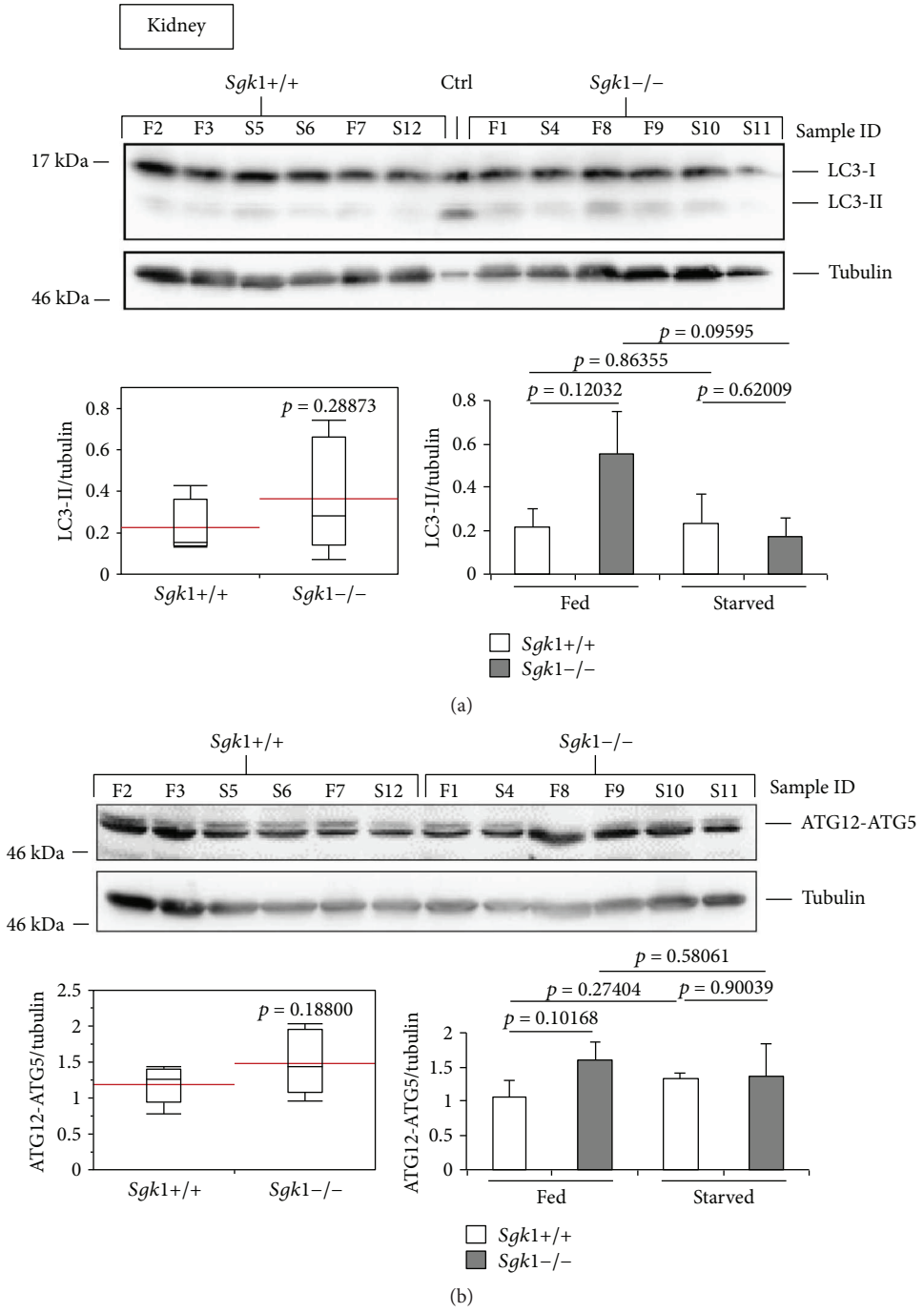

Figure 4: LC3-II and ATG12-ATG5 abundances are unaltered in the mouse kidney of Sgk1 knockout mice. LC3 lipidation (a) and ATG12-ATG5 (b) abundances were analysed by Western blotting (upper panel) and quantified (lower panels) using Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice ($n = 6$). Of those, mice were either fed (F, $n = 3$) or starved (S, $n = 3$) as indicated (sample ID). LC3-positive control (Ctrl) was used to indicate the migration of nonlipidated LC3-I and lipidated LC3-II. Red lines on the box plots (lower panel, left) represent the mean values of all 6 Sgk1 knockout (*Sgk1*−/−) and wild-type (*Sgk1*+/+) mice (lower panel, left) or with regard to fed and starved conditions (lower panel, right). *p* values are provided. Supporting data is provided (Suppl. Data Set).

apparent when we calculated (up to 1541 cells per treatment, $n = 3$) the number of GFP-WIPI1 puncta-positive cells treated either with siControl or with siSGK1 (Figure 5(b)). In both fed and starved conditions, the absence of SGK1 induced a significant increase of GFP-WIPI1-harboring autophagosomes (puncta) (Figure 5(b)). Importantly, we confirmed prominent silencing of endogenous SGK1 in all applied conditions by parallel Western blotting ($n = 6$, 3 experiments are shown in Figure 6(a)). Subsequently, we assessed the abundance of lipidated, autophagosomal LC3 (LC3-II) in both the presence (siControl) or the absence of endogenous SGK1 (siSGK1) in fed

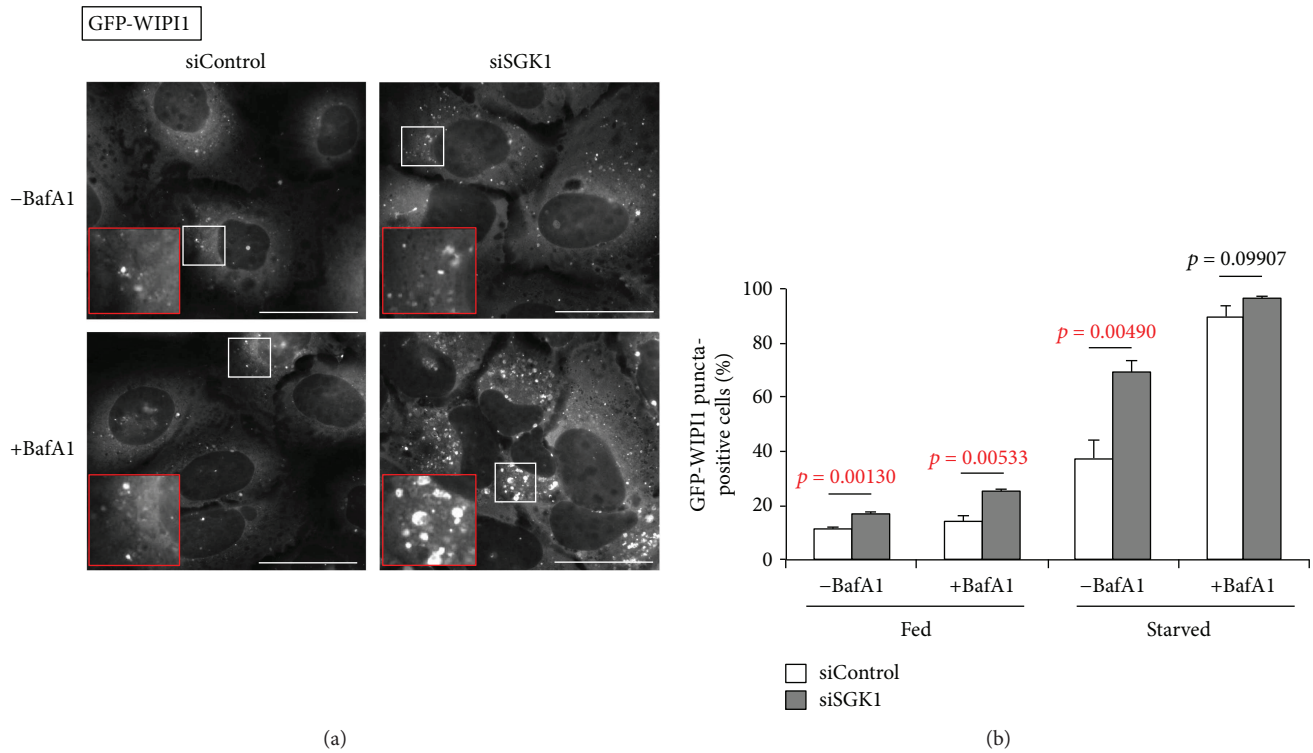

FIGURE 5: SGK1 silencing provokes an increase of GFP-WIPI1 puncta. Stable GFP-WIPI1 expressing U-2 OS cells were transiently transfected with control siRNA (siControl) or siSGK1 and fed or starved for 3 h with or without bafilomycin A1 (BafA1). (a) Representative images for starved conditions with and without BafA1 treatment are shown. Scale bars 40 μm. (b) The percentage GFP-WIPI puncta cells were calculated (up to 1541 cells per condition, $n = 3$). p values are provided. Supporting data is provided (Suppl. Data Set).

and starved conditions with or without lysosomal inhibitor (BafA1) ($n = 6$, 3 independent experiments are shown in Figure 6(a)). Again, we found that in cells with downregulated SGK1 (siSGK1), more LC3-II protein was present, in fed and also in starved conditions (Figure 6(a), lower panels; Figure 6(b)). Importantly, in conditions where we employed bafilomycin A1 (BafA1), the abundances of LC3-II further increased, demonstrating that not only more lipidated LC3 (LC3-II) was produced in the absence of SGK1, but also more autophagic flux was observed (Figure 6(b)). In addition, we assessed the abundance of ULK1 protein upon SGK1 silencing and also in parallel its phosphorylation status with regard to the inhibitory phosphorylation by TORC1 (S758) (Figure 7(a); $n = 3$ with duplicates; one of each duplicate is presented). As expected, we found that upon starvation, less ULK1 was phosphorylated at S758 (Figure 7(b), upper panel). Interestingly, we further found that ULK1 protein levels increased in the absence of SGK1 (Figures 7(a) and 7(b) lower panel), but not its phosphorylation at serine 758 (Figure 7(b)). In fact, upon starvation, significantly less ULK1 was phosphorylated in the absence of SGK1 (Figure 7(b)).

4. Discussion

To investigate the role of SGK1 in autophagy, we analysed the abundance of the autophagy markers LC3-II and ATG12-ATG5 in organ tissues derived from SGK1 knockout and wild-type mice. We also downregulated endogenous SGK1 in U-2 OS GFP-WIPI1 cells and analysed autophagic activity *in vitro*.

Our *in vivo* analysis using quantitative LC3-II and ATG12-ATG5 Western blotting suggests that in SGK1 knockout mice autophagic activity is significantly increased in red muscle tissue (Figure 1) and weakly in the white muscle and the liver (Figure 2). As increased levels of LC3-II and ATG12-ATG5 suggest increased autophagic activity, SGK1 likely inhibits autophagy in these tissues.

Our *in vitro* analysis using quantitative LC3-II Western blotting and GFP-WIPI1 puncta analysis confirmed that SGK1 should inhibit the process of autophagy. We observed a significant increase of GFP-WIPI1-bound autophagosomes (puncta). This demonstrates that the absence of SGK1 provokes the induction of autophagy via the canonical requirement for newly produced PI3P, which is then bound by WIPI1/2 proteins (here GFP-WIPI1) to mediate the recruitment of the ATG12-ATG5/ATG16L complex for subsequent LC3 lipidation (production of LC3-II) [5].

Indeed, when we assessed LC3 lipidation in cells depleted from endogenous SGK1, a significant increase of LC3-II was confirmed. This was found in both fed and starved conditions, showing that SGK1 should inhibit basal as well as induced autophagy. We further addressed the question if the absence of SGK1 would additionally provoke an increase of the autophagic flux. These assessments were carried out using fed and starved conditions in both the presence or the absence of bafilomycin A1, which inhibits lysosomal function hence blocking autolysosomal degradation. Indeed, in the absence

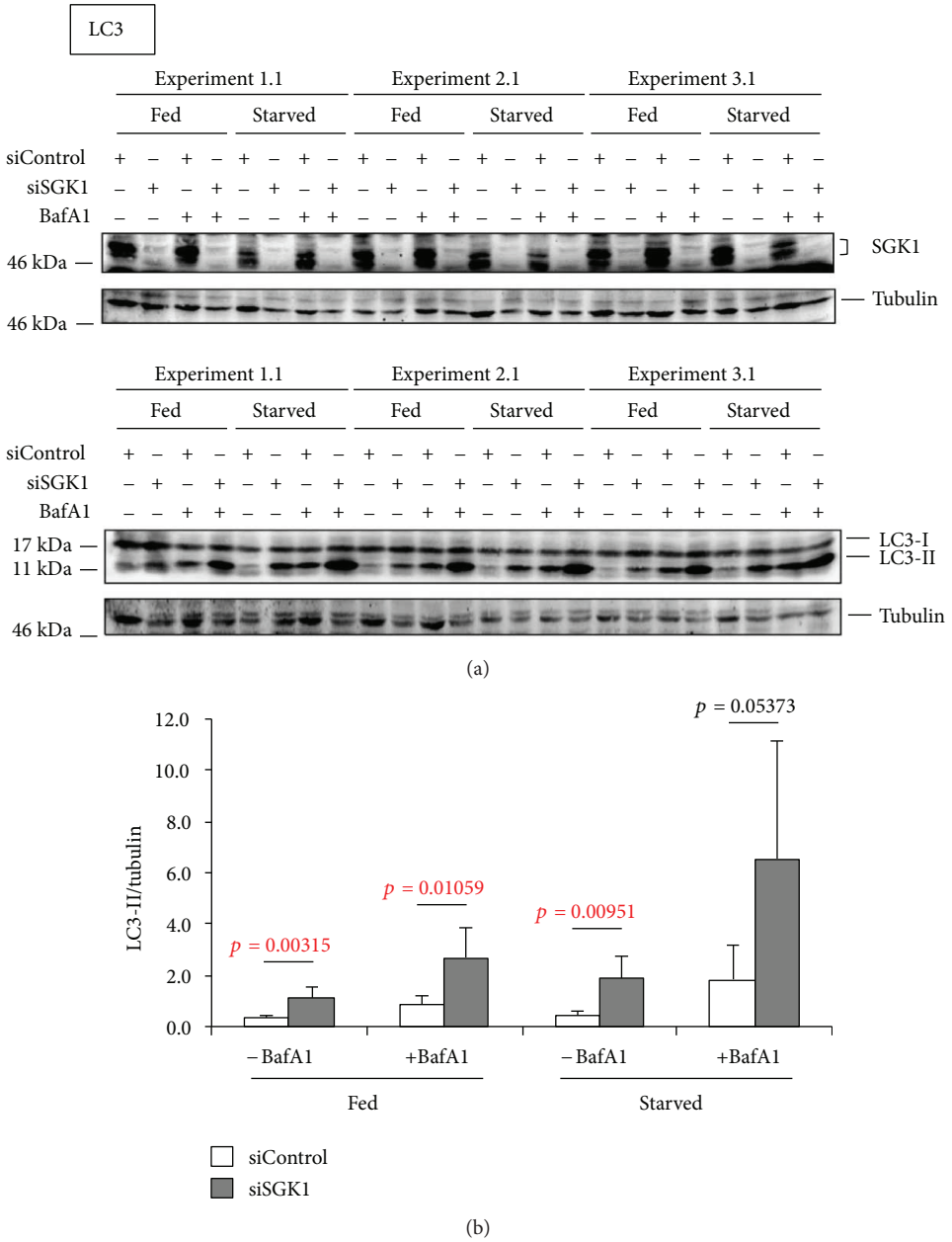

Figure 6: Elevated LC3 lipidation in SGK1-depleted cells. Stable GFP-WIPI1 expressing U-2 OS cells were transiently transfected with control siRNA (siControl) or siSGK1 and fed or starved for 3 h with or without bafilomycin A1 (BafA1). (a) SGK1 depletion was confirmed by Western blotting ($n = 6$, 3 independent experiments are shown). The autophagic flux was assessed by LC3 (LC3-II) Western blot analysis. (b) LC3-II levels were normalized over tubulin ($n = 6$, 3 experiments are shown). p values are provided. Supporting data is provided (Suppl. Data Set).

of SGK1, LC3-II levels (and also GFP-WIPI1 puncta) significantly increased, suggesting that the autophagic flux was also stimulated without SGK1. Taken together, both our *in vivo* and *in vitro* analyses of autophagy support the notion that SGK1 should function as a negative regulator of autophagosome formation and autophagic degradation.

Additionally, we also asked whether or not we could observe a difference in ULK1 protein level and its phosphorylation status in the absence of SGK1. Interestingly, we observed that upon SGK1 depletion more ULK1 protein was detected, indicating that SGK1 should contribute to the regulation of ULK1 gene expression, a topic of recent interest [31]. Moreover, as more ULK1 protein was present in the absence of SGK1, not more (but even less) phosphorylation of ULK1 at serine 758 occurred. TORC1 targets serine 758 in ULK1 for phosphorylation, resulting in the inhibition of ULK1. A decrease in ULK1 phosphorylation at serine 758 hence liberates ULK1 protein to initiate autophagy, which in fact was observed in the absence of SGK1 (see above). In general, ULK1 mRNA and protein levels are fine-tuned during the process of autophagy, showing that ULK1 availability is tightly controlled at different, as yet unidentified, stages [32].

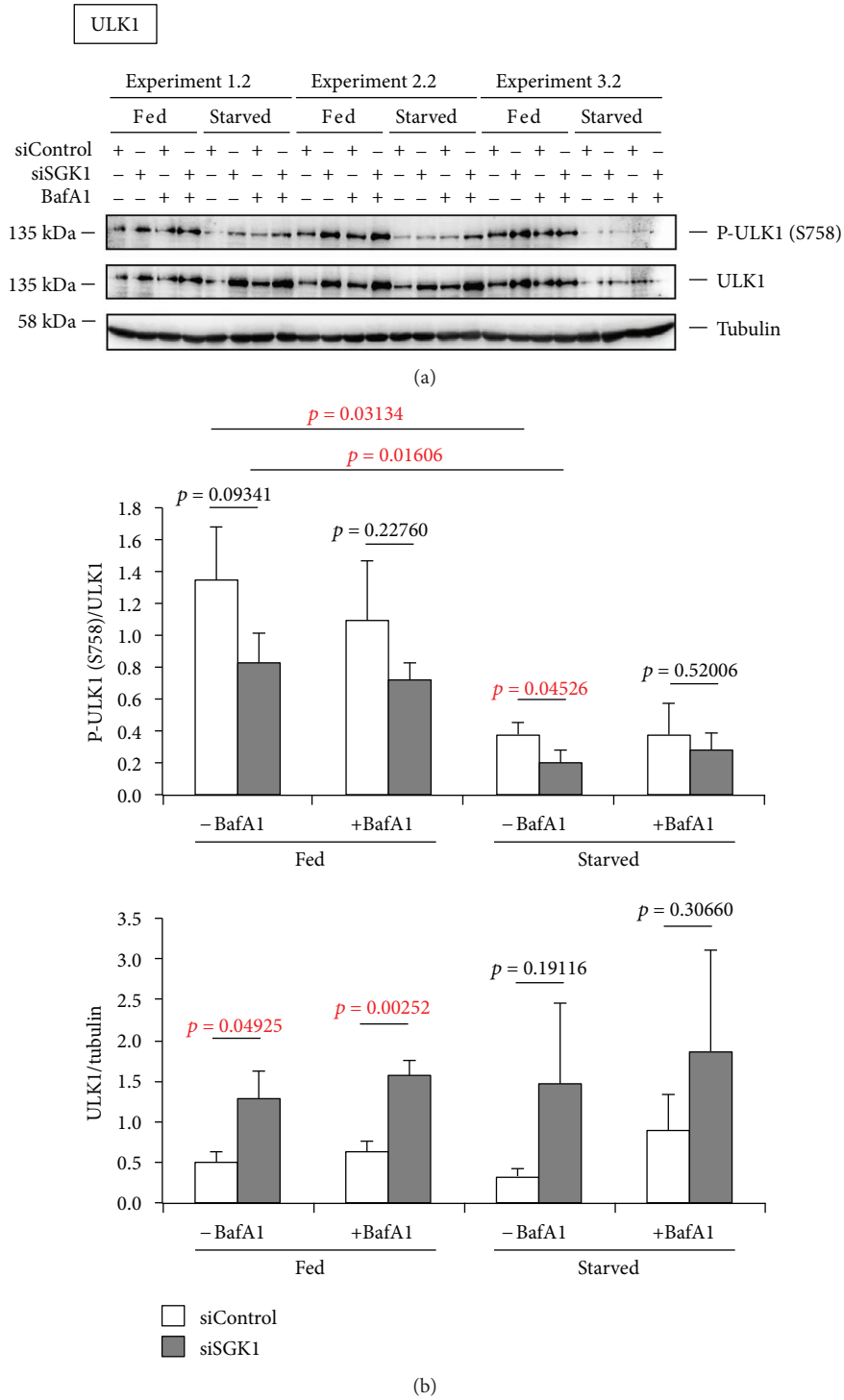

FIGURE 7: Increased ULK1 protein abundance in SGK1-depleted cells. Stable GFP-WIPI1 expressing U-2 OS cells were transiently transfected with control siRNA (siControl) or siSGK1 and fed or starved for 3 h with or without bafilomycin A1 (BafA1). (a) Phosphorylated and total ULK1 protein was detected by sequential Western blotting. (b) Phospho-ULK1 (S758)/ULK1 and ULK1/tubulin ratios were quantified ($n = 3$, in duplicates). p values are provided. Supporting data is provided (Suppl. Data Set).

We hypothesize (Figure 8) that SGK1 may regulate autophagy by contributing to regulate ULK1 protein level, perhaps through modulating ULK1 gene expression. Interestingly, the transcription factor FOXO3 was found to be specifically phosphorylated by SGK1, which results in the nuclear exclusion of FOXO3 hence its inhibition to function as a transcription factor [27, 33]. FOXO3, however, is required to initiate ULK1 gene expression as previously shown [34]. Based on this, it is tempting to speculate that SGK1 may regulate ULK1 gene expression via FOXO3 (Figure 8).

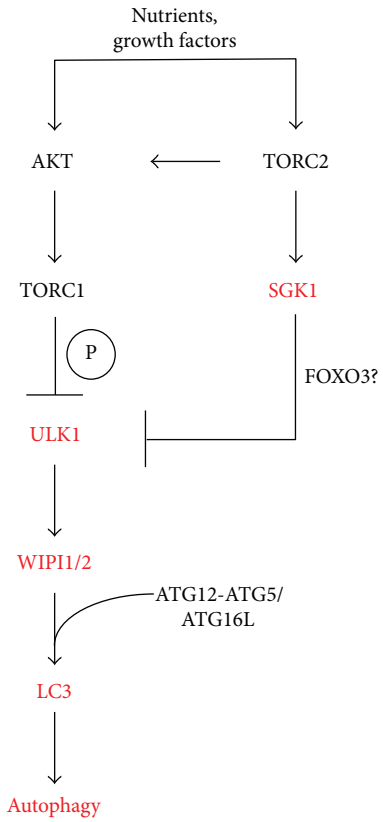

Figure 8: Working model for the role of SGK1 in the control of autophagy.

Conflicts of Interest

All authors approved the manuscript for submission and have no conflict of interest to declare.

Authors' Contributions

Jakob Voelkl bred and dissected the mice and provided isolated organ tissues. Theresia Zuleger, Julia Heinzelbecker, Zsuzsanna Takacs, and Catherine Hunter conducted the experiments. Theresia Zuleger, Julia Heinzelbecker, Zsuzsanna Takacs, and Tassula Proikas-Cezanne analysed the data and wrote the manuscript. Florian Lang and Tassula Proikas-Cezanne conceived the study.

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Supplementary Materials

Supporting data is provided in the Supplementary Data Set (Suppl. Data Set). As indicated in each figure legend, the Suppl. Data Set (excel file) includes statistical analyses with p values for the following main figures: Figures 1(a), 1(b), 2(a), 2(b), 3(a), 3(b), 4(a), 4(b), 5(b), 6(b), and 7(b). Individual excel sheet labels correspond to the main figure labels. *(Supplementary Materials)*

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Research Article

From Oxidative Stress Damage to Pathways, Networks, and Autophagy via MicroRNAs

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Oxidative stress can alter the expression level of many microRNAs (miRNAs), but how these changes are integrated and related to oxidative stress responses is poorly understood. In this article, we addressed this question by using in silico tools. We reviewed the literature for miRNAs whose expression is altered upon oxidative stress damage and used them in combination with various databases and software to predict common gene targets of oxidative stress-modulated miRNAs and affected pathways. Furthermore, we identified miRNAs that simultaneously target the predicted oxidative stress-modulated miRNA gene targets. This generated a list of novel candidate miRNAs potentially involved in oxidative stress responses. By literature search and grouping of pathways and cellular responses, we could classify these candidate miRNAs and their targets into a larger scheme related to oxidative stress responses. To further exemplify the potential of our approach in free radical research, we used our explorative tools in combination with ingenuity pathway analysis to successfully identify new candidate miRNAs involved in the ubiquitination process, a master regulator of cellular responses to oxidative stress and proteostasis. Lastly, we demonstrate that our approach may also be useful to identify novel candidate connections between oxidative stress-related miRNAs and autophagy. In summary, our results indicate novel and important aspects with regard to the integrated biological roles of oxidative stress-modulated miRNAs and demonstrate how this type of in silico approach can be useful as a starting point to generate hypotheses and guide further research on the interrelation between miRNA-based gene regulation, oxidative stress signaling pathways, and autophagy.

1. Introduction

The flux and redox chemistry of reactive oxygen species (ROS) influence key physiological responses of tissues through the capacity of being able to regulate virtually all signal transduction pathways and gene transcription factors of the cellular systems. As a consequence, disturbances of the regulatory role of ROS, often described with the generic term "oxidative stress," can lead to the development of major cellular failures that have been described as a recurring trait in the pathobiology of many, if not all, types of diseases.

The increasing interest in control mechanisms of the gene-environment interaction has stimulated a series of studies in this field, pointing to microRNA (miRNA) molecules as emerging molecular mediators of oxidative stress and ROS chemistry. Moreover, accumulating evidence points to a central role of the lysosomal degradative pathway autophagy in oxidative stress responses and in oxidative stress-related pathobiology.

This article focuses on the relations between oxidative stress, microRNAs, and autophagy. We use existing knowledge combined with in silico analyses to introduce concepts that can be useful for studying the connections between miRNA-based gene regulation, oxidative stress-induced pathways, and autophagy.

miRNAs are small noncoding RNAs, which, after a process of maturation, have a typical length of 18–25 bp. miRNAs have a unique role in posttranscriptional gene regulation. Depending on various grades of complementarities, miRNAs can cause a block in mRNA translation or even mRNA degradation. Since their discovery, miRNAs have been known to regulate the expression of a very large number of proteins, and it is supposed that they could regulate up to 30% (or even more) of the human genome [1]. Different studies show the importance of miRNAs in the regulation of processes like cell growth, differentiation, apoptosis, and carcinogenesis. Furthermore, miRNAs can be expected to play an important role in the diagnosis and prognosis of a large number of human diseases, since the quali-quantitative miRNA composition of every tissue is different depending on the state of human health [2].

In the modern way of defining oxidative stress, this adverse condition occurs when a cell or tissue is unable of controlling redox-dependent reactions and signal transduction processes by modified flux or reactivity of ROS. Depending on the intensity of this redox challenge, biomolecule damage may also occur, with accumulation of byproducts and increased need for detoxification and turnover of cellular components. The generic term "ROS" is used to comprehensively describe a series of molecules that derive from the tetravalent reduction of molecular oxygen and NO-derived metabolites, plus a series of second-generation products of their reactivity with biomolecules (lipids, proteins, and nucleic acids). Second-generation products include amongst others organic free radicals, peroxides, and reactive carbonyls, which are reported to play important roles in aging and disease development. The most relevant ROS forms in cellular systems are mainly represented by hydrogen peroxide and superoxide anion, which play important biochemical roles spanning from cell cycle regulation to the defense against pathogens during phagocytosis. Redox homeostasis, and thus the physiological control of redox-sensitive signal transduction pathways, is ensured by the activity of a battery of cellular detoxification and antioxidant enzymes.

Since miRNAs are important modulators of protein expression, the functional relationship between oxidative stress and the miRNA-dependent regulation of ROS-generating and redox-regulating enzymes, and their associated targets and pathways, is of great interest. However, the interaction between miRNAs and their molecular targets is often complex and difficult to interpret, thus introducing a major technical complication in explorative and prediction studies as well as in model interpretation. Indeed, every single gene target can be regulated by many miRNAs and every single miRNA may regulate the expression of many different target proteins [2].

Several types of dedicated software for consultation of miRNA databases are available on the web, and this helps to get a comprehensive overview of possible interactions. In recent years, different algorithms have been developed to predict the role of miRNAs expressed in different organisms, which besides humans include other vertebrates, *Drosophila melanogaster*, and plants. The database DIANA LAB [3–6] includes various algorithms that predict the association between a miRNA and its targets, analysis of expression data, and pathway attribution. Other web resources are MicroInspector [7], miRanda-mirSVR [8, 9], NBmiRTar [10], PicTar [11], Segal Lab of Computational Biology [12], RNA22 microRNA target detection [13], and TargetScan [13–15]. Another very useful web resource is miRecords [16], a collection of experimentally validated miRNA-target interactions. These resources helped us to develop a program called SID1.0 (String IDentifier) able to associate the targets and pathways of different miRNAs, and even in the opposite way, to associate them to different miRNAs [17].

In the present study, we firstly identified a small number of miRNAs already observed in the literature to be up- or downregulated after exposure of *in vitro* cultured human cells to oxidative stress. Further, using TargetScan [13–15] and DIANA LAB [6], we searched for the targets and pathways of those miRNAs implicated in oxidative stress, before employing SID 1.0 [17] for final determination of the common targets and pathways. We used miRecords [16] to identify the data already validated experimentally. Next, we searched for new miRNAs likely associated to the targets and pathways found to be involved in the oxidative stress response. Moreover, we analyzed the targets obtained from SID1.0 through the use of Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, http://www.ingenuity.com) to show the networks and biological functions likely modulated by the identified targets. One of the most dominant pathways turned out to be protein ubiquitination. Lastly, we performed a literature search for links between our identified target genes, as well as our identified candidate novel oxidative stress-related miRNAs, and autophagy.

2. Materials and Methods

2.1. Data Acquisition and SID1.0 Prediction Analysis. From the TargetScan database, we obtained the predicted target genes of the miRNAs of interest. The targets of a miRNA are indicated with a specific gene ID system (RefSeq ID). For each miRNA, a dataset (i.e., a group list of RefSeq IDs) of the predicted targeted genes was created. Since a visual inspection of the IDs would be impractical due to their large number (up to thousands of IDs), they have been automatically indexed using a simple program written in Fortran (SID1.0; String IDentifier, see http://www.fis.uniurb.it/spada/SID_minipage.html and [17]) that looks for RefSeq

IDs shared by the predicted target genes of the different datasets. SID1.0 is in fact based on an algorithm of sequential exhaustive searches that has been implemented in Fortran 90 using very elementary methods. SID1.0 performs an exhaustive search within each individual one-column ASCII input file and reports the result (i.e., the number of common targets) on an ASCII output file in the form of a table that summarizes the common IDs. Thus, the main advantage of SID1.0, which works as a filter on the information provided by the web pages hosting the miRNA databases, is that it is completely independent from the algorithms on which the databases rely. In this way, our procedure builds upon the prediction algorithms used in the databases, whose outputs are scrutinized by SID1.0. SID1.0 has been developed and tested in a Mac OS X environment and is currently compiled using the Intel Fortran 90 compiler.

Each gene in the group list has the related information in NCBI's Entrez Nucleotide database. It is possible to perform a reverse search by obtaining the miRNAs predicted to target a gene from TargetScan. For each gene, a dataset of the miRNAs predicted to target the gene was created. The names of the miRNAs were indexed using SID1.0, which looks for miRNA names shared by the predicted targeting miRNAs of the different datasets. Furthermore, for a defined miRNA name, target genes can be automatically retrieved from the DIANA-microT 3.0 database. A list of gene names or a list of RefSeq IDs is provided, and the program translates them into Ensembl IDs. The list of genes is compared to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways Database, and IDs are indexed using SID1.0, which looks for KEGG pathway IDs shared by the predicted target genes of the different datasets.

In this way, we were able to obtain the common target genes of specific miRNAs, the common targeting miRNAs of specific genes, and the common pathways of specific miRNAs.

2.2. IPA (Ingenuity Pathways Analysis). Data were analyzed by the use of Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com).

For a graphical representation of the molecular relationships between selected target molecules involved in oxidative stress, molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base.

The functional analysis identified the biological functions that were most significant to the molecules in the network. The network molecules associated with biological functions in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used in assigning each biological function to a particular network.

3. Results and Discussion

3.1. Oxidative Stress-Modulated miRNAs. From the literature, we found the following 13 miRNAs to be modulated by oxidative stress in human cultured cells: let-7f, miR-9, miR-16, miR-21, miR-22, miR-29b, miR-99a, miR-125b, miR-128, miR-143, miR-144, miR-155, and miR-200c [18–23]. For the research on oxidative stress-induced alterations of miRNA expression, many studies used H_2O_2. For example, Simone et al. [18] have shown that H_2O_2-treated AG01522 primary human fibroblasts alter their expression of let-7f, miR-16, miR-21, miR-22, miR-99a, miR-143, and miR-155. Magenta et al. [19] used human umbilical vein endothelial cells (HUVEC) treated with H_2O_2, and microRNA profiling showed an increased miR-200c expression. Sangokoya et al. [20] used different H_2O_2 concentrations to treat K562 erythroleukemia cells, which responded with increased expression of miR-144. Worth of note, in this study miR-144 was identified to, in both K562 cells and primary erythroid progenitor cells, directly regulate the activity of nuclear factor-erythroid 2-related factor 2 (Nrf2 or NFE2L2), a transcription factor and master regulator of detoxification and antioxidant responses [20].

Other studies used different agents to induce ROS generation: for example, Kutty et al. [21] used *N*-(4-hydroxyphenyl)retinamide (4HPR), a retinoic acid derivative and ROS-generating agent, and showed that 4HPR increases the expression of miR-9 in human retinal pigment epithelial (ARPE-19) cells. Luna et al. [22] induced chronic oxidative stress in HTM cells (human trabecular meshwork cells) by incubation at 40% oxygen compared to 5% oxygen control-treated cells. In response to this, miR-29b expression was decreased, and since miR-29b regulates extracellular matrix (ECM) expression it could indicate that miR-29b downregulation was responsible for increased expression of several ECM genes after oxidative stress. The combination of iron and aluminum sulfate is known to produce ROS in cultures of human brain HN cells. Lukiw and Pogue [23] isolated microRNAs from HN cells exposed to magnesium sulfate (control), aluminum sulfate, or aluminum plus iron sulfate. microRNA arrays showed that miR-9, miR-125b, and miR-128 were upregulated by metal sulfate-generated ROS.

3.2. Common Targets to Oxidative Stress-Modulated miRNAs. Using our predicting tool SID1.0 [17], we identified common target genes of the 13 oxidative stress-modulated miRNAs described in the previous paragraph (let-7f, miR-9, miR-16, miR-21, miR-22, miR-29b, miR-99a, miR-125b, miR-128, miR-143, miR-144, miR-155, and miR-200c) (Table 1 and Supplementary Table 1).

We did not find any target gene common to all oxidative stress-modulated miRNAs, but we identified 13 target genes that were common to 5, 6, or 7 of them (Table 1 and Supplementary Table 1). Using miRecords [16], we found that out of these 13 targets, 3 genes (CDC14B, NFIB, and PPARA; highlighted in italics in Table 1 and bold in Supplementary Table 1) were targets that have been experimentally validated for interaction with one of the 13 oxidative stress-modulated miRNAs. Moreover, and intriguingly, the products of these 3 genes have been described to be involved in oxidative stress damage responses. CDC14B (CDC14 cell division cycle 14 homolog B) is a member of the dual-specificity protein tyrosine phosphatase family. Its protein expression has been validated to be modulated by miR-16 and miR-15b. CDC14B is involved in cell cycle control,

Table 1: Common gene targets of microRNAs with possible role in oxidative stress. Common targets of 13 oxidative stress-modulated miRNAs: hsa-let7f (91 elements), hsa-miR-9 (936 elements), hsa-miR-16 (294 elements), hsa-miR-21 (105 elements), hsa-miR-22 (330 elements), hsa-miR-29b (158 elements), hsa-miR-99a (24 elements), hsa-miR-125b (412 elements), hsa-miR-128 (785 elements), hsa-miR-143 (263 elements), hsa-miR-144 (647 elements), hsa-miR-155 (281 elements), and hsa-miR-200c (34 elements). Listed are 13 gene targets found to be common to 5, 6, or 7 of the 13 oxidative stress-modulated miRNAs. The database used for this analysis was TargetScan [13]. The miRNA-target genes marked in italics have already been validated and described to be involved in oxidative stress responses.

Target gene	Annotation	Common miRNAs
ZNF618	Zinc finger protein 618	hsa-miR-9; hsa-miR-22; hsa-miR-125b; hsa-miR-128; hsa-miR-143; hsa-miR-144; hsa-miR-155
SH3PXD2A	SH3 and PX domains 2A	hsa-miR-9; hsa-miR-22; hsa-miR-29b; hsa-miR-143; hsa-miR-144; hsa-miR-155
TNRC6B	Trinucleotide repeat containing 6B	hsa-miR-9; hsa-miR-16; hsa-miR-29b; hsa-miR-128; hsa-miR-144; hsa-miR-22
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	let-7f; hsa-miR-9; hsa-miR-22; hsa-miR-143; hsa-miR-155
CPEB3	Cytoplasmic polyadenylation element binding protein 3	hsa-miR-9; hsa-miR-16; hsa-miR-21; hsa-miR-128; hsa-miR-144
PPARA	Peroxisome proliferator-activated receptor alpha	hsa-miR-9; hsa-miR-22; hsa-miR-21; hsa-miR-128; hsa-miR-144
CLCN5	Chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	hsa-miR-9; hsa-miR-16; hsa-miR-22; hsa-miR-128; hsa-miR-155
CDC14B	CDC14 cell division cycle 14 homolog B (*S. cerevisiae*)	hsa-miR-9; hsa-miR-16; hsa-miR-125b; hsa-miR-128; hsa-miR-144
LIFR	Leukemia inhibitory factor receptor alpha	hsa-miR-9; hsa-miR-143; hsa-miR-21; hsa-miR-128; hsa-miR-144
KCNA1	Potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)	hsa-miR-9; hsa-miR-155; hsa-miR-21; hsa-miR-128; hsa-miR-144
USP31	Ubiquitin-specific peptidase 31	hsa-miR-9; hsa-miR-16; hsa-miR-155; hsa-miR-200c; hsa-miR-144
tcag7.1228	Hypothetical protein FLJ25778	hsa-miR-9; hsa-miR-16; hsa-miR-21; hsa-miR-128; hsa-miR-144
NFIB	Nuclear factor I/B	hsa-miR-9; hsa-miR-22; hsa-miR-21; hsa-miR-128; hsa-miR-29b

Note: see Supplementary Table 1 for this table in Excel format, and see Supplementary Table 2 for a full list of gene targets found to be common to ≥2 of the 13 oxidative stress-modulated miRNAs (i.e., all possible combinations).

inducing the exit of cell mitosis and initiation of DNA replication. In response to genotoxic stress, it can translocate to the nucleoplasma to activate the ubiquitin ligase APC/C (Cdh1) and, via a number of events, promote a G2 DNA damage response checkpoint [24–26]. NFIB (nuclear factor I/B) induces in association with MYB the expression of various proteins implicated in apoptosis, cell growth, cell cycle control, and cell adhesion. NFIB is a negative regulator of miR-21, as it binds the miR-21 promoter. Interestingly, it is on the other hand the NFIB mRNA that has been validated to be regulated by miR-21, thus constituting a form of double-negative feedback system [27, 28]. PPARA (peroxisome proliferator-activated receptor alpha) is a transcription factor of the steroid hormone receptor family. It regulates the expression of target genes implicated in cell proliferation, cell differentiation, and immune and inflammation responses. It has been shown that ROS induce the expression of PPARA [29]. Further, it has been validated that the expression of PPARA is regulated by miR-22 [16].

Interestingly, 5 of the other targets found in our analysis (SH3PXD2A, CBL, ClCN5, USP31, and LIFR) have been indirectly implicated in oxidative stress responses. SH3PXD2A (SH3 and PX domains 2A, also called Tks5) has been described to link NOX (NADPH oxidases) to ROS formation [30]. CBL (Cas-Br-M (murine) ecotropic retroviral transforming sequence), a ubiquitously expressed cytoplasmic adaptor protein, is simultaneously involved in the rapid degradation of TRAIL receptors and Akt phosphorylation during TRAIL treatment. Akt catalytic activation is known to increase during metabolic oxidative stress [31, 32]. Lack of proximal tubule ClCN5 is associated with increased cell proliferation and oxidative stress in mice and men [33]. LIFR (leukemia inhibitory factor receptor alpha) and its ligands play an essential role in endogenous neuroprotective mechanisms triggered by preconditioning-induced stress [34, 35]. Ubiquitin-specific peptidase 31, USP31, has a role in the regulation of NF-κB activation (implicated in stress response) by members of the TNF receptor superfamily [36].

The remaining 5 targets identified by our prediction analysis (ZNF618, TNRC6B, CPEB3, KCNA1, and tcag7.1228; see Table 1 for annotations) are novel candidate gene

products associated with oxidative stress responses yet to be experimentally explored.

For a full list of gene targets found to be common to ≥2 of the 13 oxidative stress-modulated miRNAs (i.e., all possible combinations), see Supplementary Table 2.

3.3. Common Pathways of Oxidative Stress-Modulated MicroRNAs. Using the DIANA mirPath database (DIANA LAB), we were able to identify the common pathways of the oxidative stress-modulated miRNAs analyzed above. The analysis revealed 25 pathways that were common to all 13 oxidative stress-modulated miRNAs (Table 2 and Supplementary Table 3).

Confirming the validity of our analyses, most of these pathways are known to be involved in oxidative stress responses. Amongst the pathways predicted, many important cellular functions can be mentioned. For example, the MAPK signaling pathway entails a group of important signal transduction pathways involved in various cellular functions, including cell proliferation, differentiation, and migration. In fact, it is related to almost all of the other predicted pathways (Figure 1).

The calcium signaling pathway includes a group of events leading to increased cytosolic Ca^{2+} concentrations from extra- and intracellular (ER) sources. It is also one of the basic cellular signaling pathways implied in a wide range of cellular functions. Two pathways (cytokine-cytokine receptor interaction and TGF-beta signaling pathway) are related to cytokines, which are important intercellular messengers and regulators involved in inflammatory defenses, cell growth and differentiation, apoptosis, angiogenesis, and homeostasis. Two pathways are related to the immune system (T cell receptor signaling pathway, leukocyte transendothelial migration) and are responsible for the activation of T-lymphocytes and for the transendothelial migration of leukocytes from the blood to the tissues. Five pathways are related to the cytoskeleton, extracellular matrix, and cell-cell and cell-matrix adhesion. They include adherens junctions (cell-cell adhesion), epithelial tight junctions, focal adhesions (cell-matrix adhesion), cell adhesion molecules (selectins, cadherins, integrins, and immunoglobulins) involved in cellular adhesion, costimulation, and antigen recognition, and one actin cytoskeleton regulation pathway. Two pathways include the insulin signaling pathway, leading to glycogen synthesis and increased glucose uptake, and the related type II diabetes mellitus, leading to insulin resistance through inhibition of IRS1 functions. The GnRH signaling pathway is leading to gonadotropin-releasing hormone secretion and regulation of the production and release of the gonadotropins by the pituitary. The VEGF signaling pathway is highly important in angiogenesis and is regulating a variety of very different endothelial/epithelial processes, such as proliferation and migration of endothelial cells, promotion of epithelial survival, and vascular permeability. The Wnt signaling pathway is responsible for cell fate decisions, progenitor cell proliferation, and control of asymmetric cell division in different tissues. Two pathways are related to neuronal network development. Axon guidance is important for the development of the neuronal network, and long-term potentiation is the molecular basis for learning and memory. Six pathways are related to cancers and leukemia and more specifically to acute myeloid leukemia, prostate cancer, colorectal cancer, glioma (brain tumor), and skin cancer (melanoma and basal cell carcinoma).

Interestingly, three components of the above-mentioned pathways have been validated to be modulated by the oxidative stress-modulated miRNAs of interest: TGF-beta receptor type II (TGFBR2), implied in MAPK- and TGF-beta signaling pathways, has been validated to be regulated by miR-21 (and miR-26a). Interestingly, TGFBR2 has also been related to the production of ROS [37, 38]. CDH1 is implied in melanoma, adherens junctions, and cell adhesion molecules. It has been validated that CDH1 is regulated by miR-9, and another study has shown that expression of CDH1 is downmodulated after ROS exposure [39, 40]. The forkhead box O protein 1 (FOXO1) is a tumor suppressor implied in prostate cancer and the insulin signaling pathway. It has been validated that FOXO1 is downregulated by miR-9, miR-27, miR-96, miR-153, miR-182, miR-183, and miR-186. On the other hand, two other studies showed induction of FOXO expression upon oxidative stress [41–43].

Also of note, one of our predicted common targets of oxidative stress-modulated miRNAs (Table 1), namely, CBL, is the component of three of the KEGG pathways identified (the insulin, T cell receptor, and ErbB signaling pathways).

For a full list of all KEGG pathways common to ≥2 of the 13 oxidative stress-modulated miRNAs, see Supplementary Table 4.

3.4. New Candidate MicroRNAs Potentially Involved in Oxidative Stress Responses. We inserted the common gene targets from Table 1 into the TargetScan database [13–15] to export the miRNAs modulating each of them. Using SID1.0 [17] to find the common miRNAs, we identified new candidate miRNAs that may be involved in oxidative stress responses. As shown in Table 3 and Supplementary Table 5, miR-9 was found to be common to all the 13 targets analyzed, while the 26 other miRNAs indicated in the tables were common to 9, 10, 11, or 12 of them.

Six of the identified miRNAs have already been described to be modulated during oxidative stress responses: miR-9, miR-16, miR-29b, miR-128, miR-144, and miR-200c (highlighted in italics in Table 3 and bold in Supplementary Table 5). The other 21 miRNAs that our analysis identified have not yet been ascribed a direct role in oxidative stress, and they are therefore novel candidate oxidative stress response-related miRNAs. Biological functions of several of these miRNAs have been reported. miR-101 has been the object of many studies and is well known to be involved in Akt signaling and the MAPK pathway. Moreover, miR-101 has been described to be related to various cancers and to target various tumor-suppressor genes and oncogenes, as well as to be involved in cell proliferation, migration, invasion, angiogenesis, and cell death. miR-429, miR-15, miR-195, miR-93, and miR-497 have been implicated in carcinogenesis. miR-124 is a well-studied miRNA, which seems to function as a tumor suppressor and to be implicated in cell differentiation (amongst others closely

Table 2: Common pathways (KEGG) of microRNAs associated with oxidative stress. Common pathways (KEGG pathway IDs) of hsa-let7f (129 elements), hsa-miR-9 (140 elements), hsa-miR-16 (117 elements), hsa-miR-21 (64 elements), hsa-miR-22 (101 elements), hsa-miR-29b (126 elements), hsa-miR-99a (40 elements), hsa-miR-125b (126 elements), hsa-miR-128 (103 elements), hsa-miR-143 (80 elements), hsa-miR-144 (110 elements), hsa-miR-155 (70 elements), and hsa-miR-200c (113 elements). The pathways are common to all the 13 oxidative stress-modulated miRNAs. The KEGG pathway name (first column), the gene symbols involved in each pathway (second column), and the ID of the pathway used by the KEGG database (third column) are indicated. The database used for this analysis was DIANA-MicroT 3.0 [3].

KEGG pathway name	Gene symbol	KEGG pathway ID
MAPK signaling pathway	FGF12, PRKCA, MAP3K1, RPS6KA4, SOS1, MAP3K3, SRF, PAK2, MAP2K7, FGF18, RAP1B, MAPKAPK2, ACVR1B, TGFBR2, FGF5, DUSP6, ACVR1C, PDGFRB	hsa04010
Melanoma	FGF12, FGF18, FGF5, CDH1, IGF1, PDGFRB, PIK3R3, PDGFC	hsa05218
Colorectal cancer	RALGDS, SOS1, TCF7, ACVR1B, TGFBR2, ACVR1C, DCC, PDGFRB, PIK3R3, SMAD4	hsa05210
Glioma	PRKCA, SHC2, SOS1, SHC1, IGF1, PDGFRB, PIK3R3	hsa05214
Adherens junction	CTNNA1, ACTN2, VCL, TCF7, BAIAP2, SRC, ACVR1B, SSX2IP, TGFBR2, ACVR1C, CDH1, SMAD4	hsa04520
Focal adhesion	ITGB4, PRKCA, SHC2, ACTN2, TNC, DIAPH1, VCL, COL5A1, ITGA6, SOS1, VAV3, SHC1, SRC, PAK4, PAK2, PAK6, THBS2, RAP1B, IGF1, PDGFRB, PIK3R3, PDGFC	hsa04510
TGF-beta signaling pathway	ID4, SMURF1, INHBB, THBS2, ACVR1B, TGFBR2, ACVR1C, SMAD4	hsa04350
mTOR signaling pathway	TSC1, ULK2, IGF1, PIK3R3, EIF4E	hsa04150
Prostate cancer	CCNE2, SOS1, TCF7, FOXO1, CREB5, IGF1, PDGFRB, PIK3R3, PDGFC, CREB3L2	hsa05215
Wnt signaling pathway	WNT4, PRKCA, TCF7, VANGL1, PSEN1, PPARD, SMAD4	hsa04310
Cytokine-cytokine receptor interaction	LEP, CNTFR, LIFR, CXCL11, INHBB, KITLG, TNFRSF21, ACVR1B, TGFBR2, PDGFRB, PDGFC	hsa04060
Basal cell carcinoma	WNT4, TCF7, PTCH1	hsa05217
Type II diabetes mellitus	SOCS4, PIK3R3	hsa04930
Cell adhesion molecules (CAMs)	SDC2, CLDN14, SDC1, NFASC, ITGA6, NEO1, ALCAM, NLGN2, CDH1	hsa04514
Regulation of actin cytoskeleton	ITGB4, MYH9, FGF12, ACTN2, DIAPH1, VCL, ARPC1A, ARHGEF7, PIP4K2B, ITGA6, SOS1, VAV3, BAIAP2, DIAPH2, PAK4, PAK2, PAK6, FGF18, FGF5, SLC9A1, PDGFRB, PIK3R3	hsa04810
Long-term potentiation	PRKCA, RAP1B	hsa04720
Insulin signaling pathway	TSC1, SHC2, SOCS4, CBL, SOS1, FOXO1, SHC1, PIK3R3, EIF4E	hsa04910
Leukocyte transendothelial migration	CTNNA1, PRKCA, ACTN2, CLDN14, VCL, VAV3, RAP1B, PIK3R3	hsa04670
Tight junction	CTNNA1, MYH9, PPP2R4, PRKCA, ACTN2, CLDN14, AMOTL1, SRC, CSDA	hsa04530
Axon guidance	SRGAP3, PLXNA2, NTNG1, EPHB2, SEMA6D, EPHA7, NRP1, PAK4, PAK2, PAK6, DCC, EPHB4, EFNA1	hsa04360
Calcium signaling pathway	PRKCA, SLC8A1, ADCY9, PDGFRB	hsa04020
T cell receptor signaling pathway	CBL, SOS1, VAV3, PAK4, PAK2, PAK6, PIK3R3	hsa04660
GnRH signaling pathway	PRKCA, MAP3K1, SOS1, MAP3K3, SRC, ADCY9, MAP2K7	hsa04912
ErbB signaling pathway	PRKCA, SHC2, CBL, SOS1, SHC1, SRC, ABL2, PAK4, PAK2, PAK6, MAP2K7, PIK3R3	hsa04012
Acute myeloid leukemia	SOS1, TCF7, JUP, PPARD, PIK3R3	hsa05221
VEGF signaling pathway	PRKCA, SHC2, SRC, MAPKAPK2, PIK3R3	hsa04370

Note: see Supplementary Table 3 for this table in Excel format, and see Supplementary Table 4 for a full list of all KEGG pathways common to ≥2 of the 13 oxidative stress-modulated miRNAs.

linked to neuronal differentiation) as well and in regulation of the cytoskeleton. miR-17 seems to be implied in leukemia and lung cancer. It suppresses apoptosis and regulates MAP14. miR-106b targets PTEN and has a prooncogenic function when overexpressed, by also suppressing Bim and p21 expression. miR-519 reduces cell proliferation. miR-27a is involved in cell cycle regulation and is linked with leukemia and carcinogenesis;

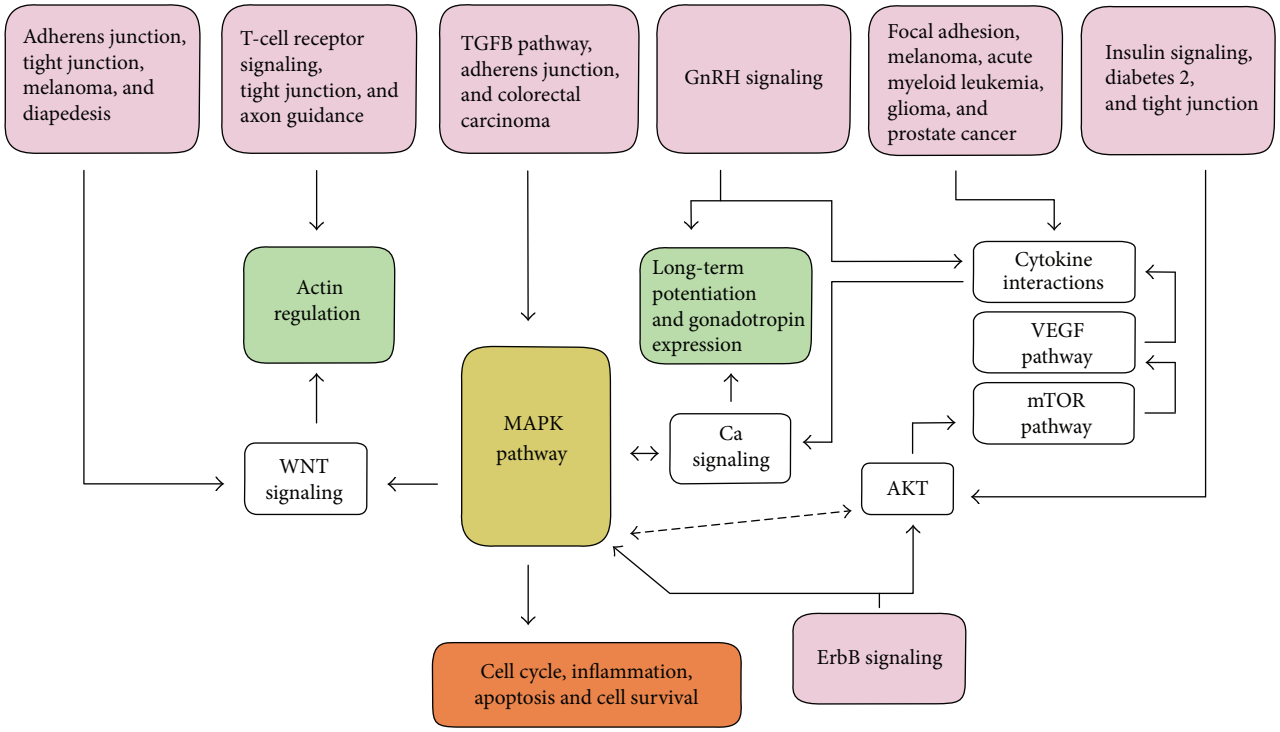


Figure 1: Simplified flow diagram indicating the interrelation between pathways predicted to be commonly modified by the 13 oxidative stress-related miRNAs considered in this study.

its downregulation inhibits cell proliferation. miR-23b is involved in carcinogenesis and cell migration, and miR-23a regulates cardiac hypertrophy. miR-424 promotes angiogenesis. Of note, a study by Li et al. screened for miRNAs altered during stress-induced premature cell senescence (which may be related to oxidative stress) and identified several of the miRNAs that are in our list as novel candidate oxidative stress response-related miRNAs (miR-15a/b, miR-106a/b, miR-20a, and miR-195) [44].

Interestingly, by using the TargetScan prediction tool, we found that Nrf2 could be modulated by several of the miRNAs that we have predicted to be involved in oxidative stress responses (Table 3), namely, miR-128, miR-144, miR-548n, miR-101, miR-23a/b, miR-27a/b, miR-106a/b, miR-17, miR-20a/b, and miR-93.

For a full list of all miRNAs that target ≥2 of the 13 gene targets of oxidative stress-modulated miRNAs, see Supplementary Table 6.

Taken together, all these pieces of information add new and interesting suggestions on the miRNA-dependent regulation of gene networks and cellular processes related to the oxidative stress response. This can be used to generate novel and testable hypotheses, as well as in the planning of further levels of experimental investigation. The bioinformatics approach shown in this paper takes into account the complexity of the regulatory interactome of individual miRNAs with the range of target genes involved in cellular pathways, comparing at the same time different miRNA hubs and regulatory subnetworks (Figure 2) during the biological/functional interpretation of the retrieved information, which is further discussed in the next section.

3.5. Biological and Functional Interpretation of Oxidative Stress-Associated MicroRNAs. The Ingenuity Pathways Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) is a useful resource to perform a functional analysis of gene targets identified during laboratory or in silico investigations, providing biological interpretations of complex events or matrices of data, which is the case in the exploration of cellular networks of molecular and functional interactions. In our case, the gene targets of miRNAs involved in oxidative stress identified by SID1.0 analysis were explored with this web tool to identify possible biological functions (Figures 3 and 4).

The resulting network indicates that the predicted gene targets of miRNAs involved in oxidative stress are associated with a few discrete common pathways. Firstly and most importantly, almost all targets are connected and flow in the "protein ubiquitination pathway." The protein ubiquitination pathway is implied in the degradation of regulatory proteins involved in a variety of cellular processes, such as cell cycle control, cell proliferation, apoptosis, DNA repair, transcriptional regulation, cell surface receptors, ion channel regulation, and antigen presentation. In fact, the IPA analysis of our data associated the obtained results with the network of cell-cell communication mechanisms, cell cycle regulation, and cellular development. The main targets in relation to these cellular responses, NFIB [45], LIFR [46], PPARα [47], and CBEP3 [48], are important regulatory proteins, which may undergo modulation effects by the ubiquitination pathway. The other top molecular and cellular functions in our functional analysis suggested possible effects of oxidative stress on cellular morphology, cellular

Table 3: miRNAs predicted to be involved in oxidative stress responses. Identification of miRNAs predicted to simultaneously target the genes identified in Table 1: ZNF618 (114 elements), SH3PXD2A (135 elements), TNRC6B (329 elements), CBL (740 elements), CPEB3 (178 elements), PPARA (933 elements), CLCN5 (167 elements), CDC14B (64 elements), LIFR (553 elements), KCNA1 (549 elements), USP31 (93 elements), tcag7.1228 (210 elements), and NFIB (281 elements). Shown are miRNAs (column 1) common to ≥9 of the 13 gene targets with the corresponding annotation. We found one miRNA (hsa-miR-9) common to all 13 gene targets. The database used for this analysis was TargetScan [13]. The miRNAs marked in italics have already been described to be involved in oxidative stress response.

Common miRNAs	Target genes
hsa-miR-9	CBL; CPEB3; PPARA; CLCN5; CDC14B; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-548c-3p	CBL; CPEB3; PPARA; CDC14B; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-128	CBL; CPEB3; PPARA; CLCN5; CDC14B; LIFR; KCNA1; ZNF618; tcag7.1228; NFIB; TNRC6B
hsa-miR-144	CBL; CPEB3; PPARA; CDC14B; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; SH3PXD2A; TNRC6B
hsa-miR-548n	CPEB3; PPARA; CLCN5; CDC14B; LIFR; KCNA1; USP31; ZNF618; NFIB; SH3PXD2A; TNRC6B
hsa-miR-655	CBL; CPEB3; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-548p	CBL; CPEB3; PPARA; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; TNRC6B
hsa-miR-101	CBL; CPEB3; PPARA; LIFR; KCNA1; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
*hsa-miR-29*a/*b*/c	CBL; CPEB3; PPARA; CLCN5; LIFR; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-195	CBL; CPEB3; PPARA; CLCN5; CDC14B; KCNA1; USP31; tcag7.1228; TNRC6B
hsa-miR-16	CBL; CPEB3; PPARA; CLCN5; CDC14B; KCNA1; USP31; tcag7.1228; TNRC6B
hsa-miR-424	CBL; CPEB3; PPARA; CLCN5; CDC14B; KCNA1; USP31; tcag7.1228; TNRC6B
hsa-miR-15a/b	CBL; CPEB3; PPARA; CLCN5; CDC14B; KCNA1; USP31; tcag7.1228; TNRC6B
hsa-miR-497	CBL; CPEB3; PPARA; CLCN5; CDC14B; KCNA1; USP31; tcag7.1228; TNRC6B
hsa-miR-23a/b	CBL; PPARA; LIFR; KCNA1; USP31; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-27a/b	CPEB3; PPARA; CLCN5; CDC14B; LIFR; KCNA1; USP31; NFIB; TNRC6B
hsa-miR-519a/b-3p/c-3p	CPEB3; PPARA; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-106a/b	CPEB3; PPARA; LIFR; KCNA1; USP31; ZNF618; NFIB; SH3PXD2A; TNRC6B
hsa-miR-17	CPEB3; PPARA; LIFR; KCNA1; USP31; ZNF618; NFIB; SH3PXD2A; TNRC6B
hsa-miR-20a/b	CPEB3; PPARA; LIFR; KCNA1; USP31; ZNF618; NFIB; SH3PXD2A; TNRC6B
hsa-miR-93	CPEB3; PPARA; LIFR; KCNA1; USP31; ZNF618; NFIB; SH3PXD2A; TNRC6B
hsa-miR-590-3p	PPARA; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-124	CBL; PPARA; CDC14B; KCNA1; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-506	CBL; PPARA; CDC14B; KCNA1; ZNF618; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
hsa-miR-513a-3p	CBL; PPARA; LIFR; KCNA1; USP31; ZNF618; tcag7.1228; SH3PXD2A; TNRC6B
hsa-miR-429	CBL; CDC14B; LIFR; KCNA1; USP31; tcag7.1228; NFIB; SH3PXD2A; TNRC6B
*hsa-miR-200*b/*c*	CBL; PPARA; CDC14B; LIFR; KCNA1; USP31; tcag7.1228; NFIB; SH3PXD2A

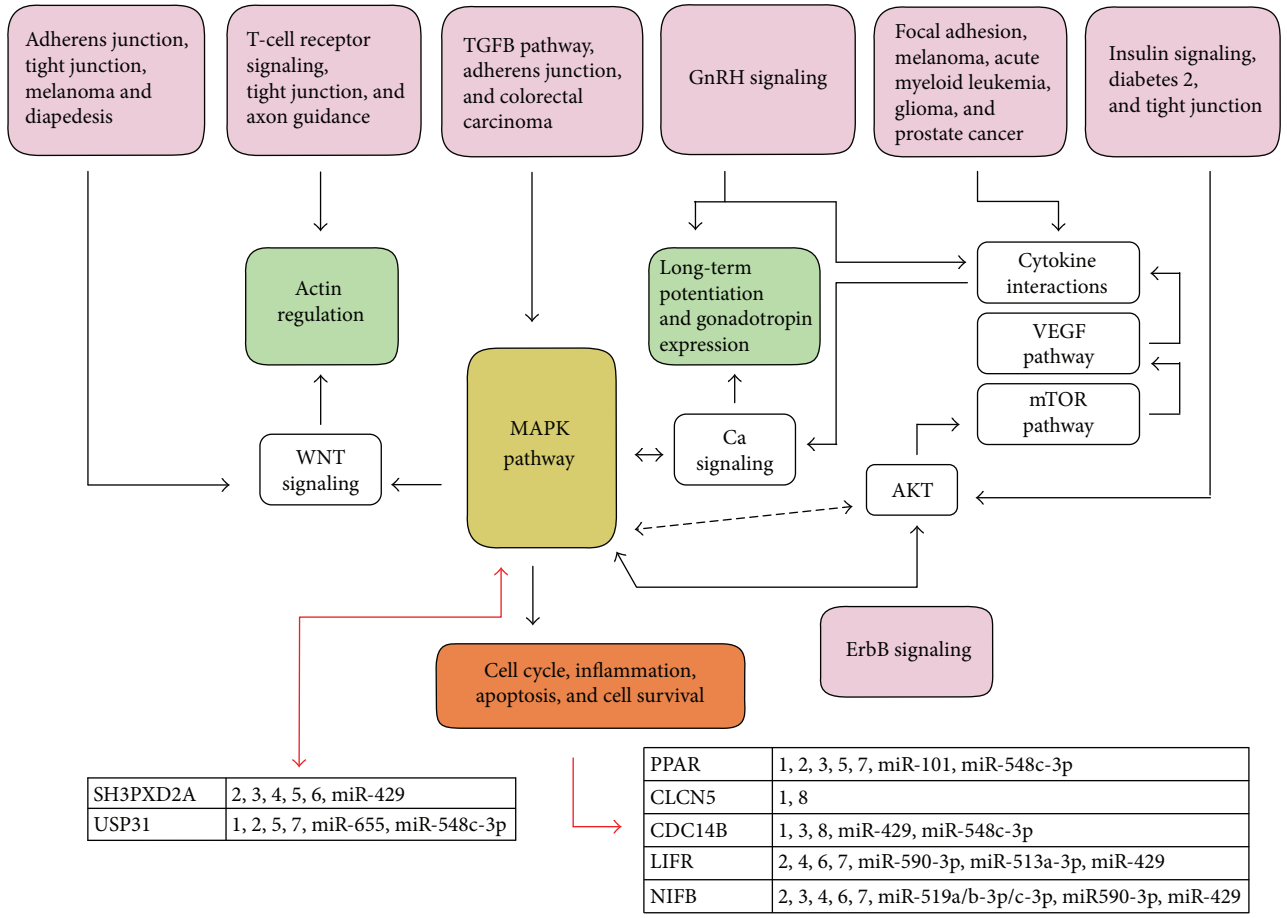
Note: see Supplementary Table 5 for this table in Excel format, and see Supplementary Table 6 for a full list of miRNAs that target ≥2 of the 13 gene targets.

assembly and organization, cellular function and maintenance, cellular movement, and energy production.

Our analyses predict that oxidative stress can lead to miRNA-mediated deregulated expression of three membrane proteins: CLCN5, KCNA1, and LIFR (Figure 4). The first two are ion transporters/channels. CLCN5 is an antiport system for chloride and protons, which is important for endosome acidification and renal tubular function [49]. KCNA1 is a voltage-gated potassium channel engaged in cell communication in the brain [50]. LIFR is a membrane protein and polyfunctional cytokine that affects cell differentiation, survival, and proliferation [46]. The transcriptional factors PPARα and NFIB are engaged in lipid metabolism (PPARα) and adhesion, cell cycle control, and cell growth (NFIB). The function of ZNF618 is unknown, but it is also associated with transcriptional regulation. The dual specificity protein tyrosine phosphatase CDC14B is involved in cell cycle control and mitotic exit due to dephosphorylation of the tumor suppressor protein p53, which is further involved in the oxidative DNA damage response [51]. Interestingly, some of the identified gene targets, such as TNRC6B and CBEP3, may participate in the functional modulation of mRNAs, thus pointing to discrete effects in the miRNA-induced translational repression of groups of genes that are likely to respond to conditions of cellular oxidative stress. In more detail, TNRC6B is itself an essential component for the translational repression mediated by miRNAs and siRNAs. TNRC6B is recruited to miRNA targets through an interaction between its N-terminal domain and an Argonaute protein; TNRC6B then promotes translational repression and/or degradation of miRNA targets through a C-terminal silencing domain [52]. CBEP3 is a RNA-binding protein that represses translation of its target mRNAs and negatively regulates EGFR signaling in neurons.

SH3PXDA2A is an adapter protein involved in invadopodia, podosome formation, extracellular matrix degradation, and invasiveness of some cancer cells. It binds matrix metalloproteinases, NADPH oxidases (NOX), and phosphoinositides and acts as an organizer protein that allows NOX1- or NOX3-dependent ROS generation and cellular localization [53].

Finally, the protein ubiquitination pathway seems to be targeted by oxidative stress-modulated miRNAs at different levels. On the one hand, 6 of the 13 identified miRNA targets



SH3PXD2A	2, 3, 4, 5, 6, miR-429
USP31	1, 2, 5, 7, miR-655, miR-548c-3p

PPAR	1, 2, 3, 5, 7, miR-101, miR-548c-3p
CLCN5	1, 8
CDC14B	1, 3, 8, miR-429, miR-548c-3p
LIFR	2, 4, 6, 7, miR-590-3p, miR-513a-3p, miR-429
NIFB	2, 3, 4, 6, 7, miR-519a/b-3p/c-3p, miR590-3p, miR-429

Figure 2: MicroRNAs predicted to target genes involved in the pathways modulated by oxidative stress. We added to the previous simplified flow diagram (shown in Figure 1) the miRNAs that we predicted to be putative novel actors in oxidative stress responses (shown in Table 3) and grouped them according to their common gene targets and overall relationship to cellular pathways/responses. 1 = miR-195, miR-424, miR-15a/b, and miR-497; 2 = miR-106a/b, miR-17, miR-20a/b, miR-93, and miR-519d; 3 = miR-124 and miR-506; 4 = miR-655, miR-548c-3p, and miR-101; 5 = miR-519a/b-3p/c-3p, miR-590-3p, and miR-513a-3p; 6 = miR-548n, miR-23a/b, and miR-27a/b; 7 = miR-548p and miR-429; and 8 = miR-548n and miR-27a/b.

(PPARα, LIFR, CLCN5, NFIB, CBEP3, and SH3PXD2A) are known to be ubiquitin C substrates, whereas on the other hand CBL and USP31 are members of the ubiquitination pathway itself. USP31 is involved in recognition and processing of polyubiquitin precursors as well as of ubiquitinated proteins. CBL, a RING finger E3 ubiquitin ligase, is required to convey substrates to proteasomal degradation, mediating the transfer of ubiquitin from ubiquitin-conjugating enzymes to specific substrates; at the same time, it is known to be a negative regulator of many signal transduction pathways [54].

Intriguingly, many of the miRNAs shown in Table 3 have been demonstrated to be able to target enzymes involved in the ubiquitination process. miR-9 targets CBL (E3 ubiquitin ligase) [55]. miR-16 and miR-128 downregulate translation of the Smurf2 protein, a tumor-suppressive ubiquitin ligase [56]. miR-101 targets MARCH7, a member of the RING finger protein family of E3 ubiquitin ligases. miR-429 and miR-200 are involved in the expression of various ULM (ubiquitin-like modifiers) proteins [57]. miR-497 is a negative regulator SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) [58], and miR-17 negatively regulates TNF-α signaling by acting on the modulation of the protein ubiquitin processes [59]. miR-93 directly suppresses ubiquitin ligase b-TRCP2 expression [60], and miR-124 was found to directly influence the activity of ubiquitin-specific proteases (USP) 2 and 14 [61, 62].

3.6. The Predicted Oxidative Stress-Modulated miRNAs That Affect the Protein Ubiquitination Pathway Also Regulate Autophagy. Autophagy dysfunction has been observed in various human pathologies such as cancer, autoimmune and infectious diseases, and neurodegenerative disorders. Ischemia and oxidative stress cause protein aggregation and mitochondrial dysfunction. On one side, upstream processes such as protein misfolding and aggregation lead to autophagy induction, whereas on the other side autophagy may fail if protein aggregation is very extreme.

Oxidative damage can irreversibly modify proteins, so that they need to be degraded. Two proteolytic systems execute the degradation task: the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal system. The proteasome needs to unfold and linearize proteins in order to do

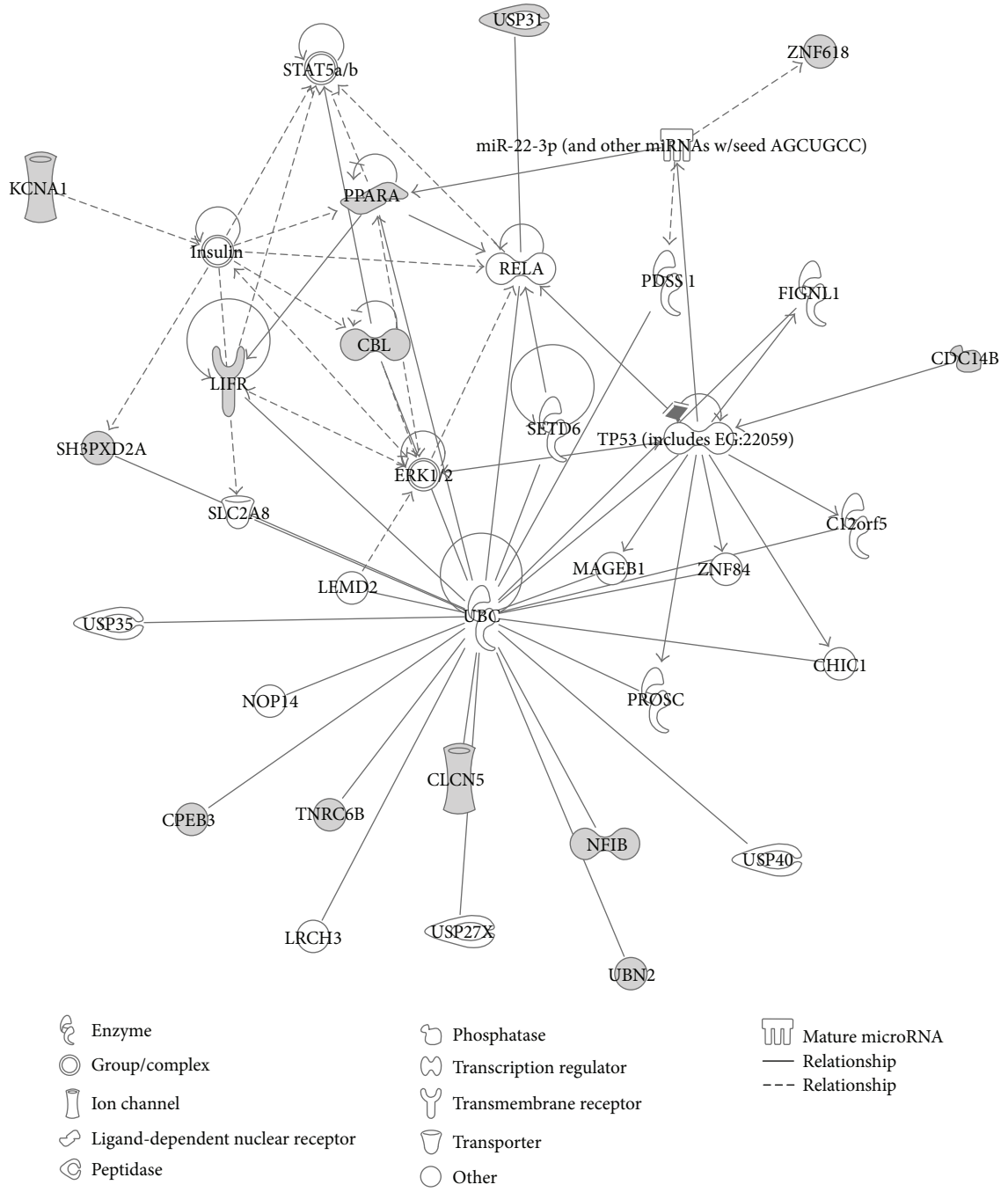


Figure 3: Graphical representation of the molecular relationships between oxidative stress-modulated miRNA gene targets. Indirect interactions exist between the protein products of the 13 gene targets of oxidative stress-modulated miRNAs predicted from our SID1.0 analysis (shown in Table 1). Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The dotted edges indicate indirect interactions whereas the others indicate direct interactions. All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product, as indicated in the legend. The filled grey nodes indicate the 13 target molecules obtained from our SID1.0 analysis.

its job (since the polypeptides need to be threaded through the very narrow core of the proteasome), and therefore the UPS cannot degrade irreversibly misfolded or aggregated proteins. The autophagic process, on the other hand, can degrade proteins of any size or form, since the material that is to be degraded is collected into double- or multimembrane vacuoles termed "autophagosomes," which are very large compared to proteins, and often can have a diameter of 500–1500 nm. Thus, autophagy can degrade not only macromolecules but also even large organelles, including

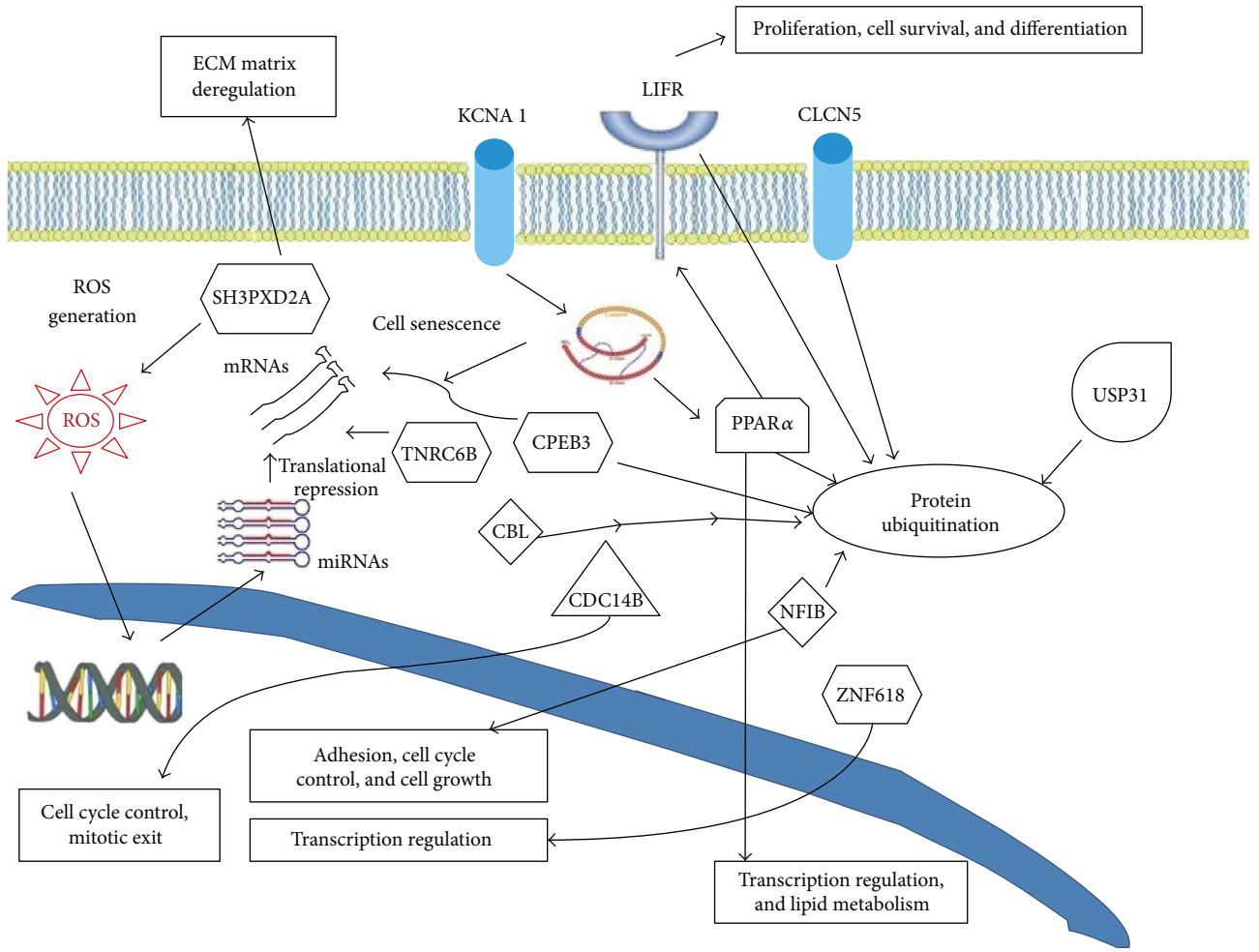


Figure 4: Simplified overview of some of the effects that our identified oxidative stress-modulated miRNA gene targets may generate and their relation to protein ubiquitination.

mitochondria. Interestingly, ubiquitin is used as a "degrade me" recognition signal not only in the ubiquitin-proteasome system but also in many forms of autophagy. The underlying mechanistic principle for this lies in ubiquitin-binding subtypes of the so-called autophagy receptors (e.g., p62/SQSMT1, NBR1, OPTN, TOLLIP, Cue5, NDP52, and TAX1BP1). These autophagy receptors have one protein domain that binds to ubiquitinated cargo and another domain that interacts with a class of small proteins that are attached to the autophagic membrane (Atg8 family proteins). Thereby, the cargo is recruited to the forming autophagosome (the "phagophore") and eventually sequestered inside it after the phagophore has closed. Upon fusion of the autophagosome with lysosomes, the inner autophagosomal membrane and the sequestered material are degraded by lysosomal enzymes, and the building blocks are recycled into the cytoplasm for reuse by the cell. Unlike the UPS, which only degrades ubiquitinated proteins, the ubiquitin "degrade me" signal is used to initiate the autophagic degradation of a variety of cellular structures, including mitochondria, RNA granules, protein aggregates, bacteria, the midbody, and even proteasomes [63]. Damaged mitochondria can be ubiquitinated at multiple outer mitochondrial membrane proteins and recognized by the autophagy receptors p62/SQSMT1, OPTN, NDP52, and TAX1BP1, whereas ubiquitinated protein aggregates can be targeted for autophagic degradation via binding to the autophagy receptors p62/SQSMT1, NBR1, OPTN, TOLLIP, and Cue5 [63]. In sum, ubiquitin-dependent autophagy can serve an important role in helping cells to neutralize damage caused by oxidative stress.

Given the established role of ubiquitin in autophagy, and the fact that our integrated in silico analysis suggested that the effects of oxidative stress-related miRNAs intersect at the protein ubiquitination pathway, we reasoned that these miRNAs may also converge to regulate autophagy. Indeed, through a literature search, we found that in fact all the oxidative stress-related miRNAs that we had identified by the reverse approach shown in Table 3 and that target enzymes involved in the ubiquitination process (miR-9, miR-16, miR-17, miR-93, miR-101, miR-124, miR-128, miR-200, miR-429, and miR-497) have been reported to regulate autophagy [64–76]. For example, miR-9 affects autophagy by targeting the products of the essential autophagy-related

(ATG) genes ATG5 [68] and Beclin1 [75], whereas miR-17 targets ATG7 [66], Beclin1 [64], and p62/SQSTM1 [70]. miR-124 targets Beclin1 [74] and p62/SQSTM1 [71]. Modulation of this set of miRNAs that we identified by in silico analysis (miR-9, miR-16, miR-17, miR-93, miR-101, miR-124, miR-128, miR-200, miR-429, and miR-497) thus appears to have a strong potential to concertedly mediate the effects of oxidative stress on both protein ubiquitination and autophagy. This illustrates how our in silico approach can lead to specific predictions and hypotheses that can be further tested experimentally.

3.7. Additional Links between Oxidative Stress-Modulated miRNAs and Autophagy. Autophagy, including selective autophagy, can in many cases operate independently of ubiquitination [63]. We therefore also performed literature searches for links between autophagy and the other candidates from our list of predicted oxidative stress-modulated miRNAs shown in Table 3. We found that several of these miRNAs have been implicated in autophagy regulation, that is, miR-15a, miR-20a/b, miR-23a/b, miR-29a, miR-106a/b, miR-195, and miR-590-3p [67, 70, 73, 77–81]. For example, miR-29a targets the product of the essential autophagy-related gene ATG9A as well as that of the master transcriptional regulator of lysosomal biogenesis and autophagy, TFEB [77]. As another example, miR-195 has been shown to target GABARAPL1 (of the Atg8 family) [78] and ATG14 [79].

Finally, we examined if we could identify links between autophagy and gene products from our predicted set of common gene targets of oxidative stress-modulated miRNAs shown in Table 1. We found that at least two of the target genes have been firmly demonstrated to play a role in autophagy, namely, CBL and PPARα. Interestingly, CBL can act as an autophagy receptor to deliver Src (a nonreceptor tyrosine kinase) and paxillin (an adapter protein of focal adhesion complexes) for autophagosomal degradation [82, 83]. Of note, CBL performs this function independently of its E3 ligase activity [82, 83]. PPARα has been demonstrated to control the transcription of a large number of autophagy-related genes in starved hepatocytes [84]. Moreover, fenofibrate, which is a potent agonist of PPARα that often also induces ROS, was shown to induce autophagy-dependent degradation of KEAP1, which again led to increased activity of Nrf2 and protection against oxidative stress damage [85].

Altogether, these identified links indicate that oxidative stress-modulated miRNAs may affect autophagy in a broad sense (i.e., both ubiquitin-dependent and ubiquitin-independent autophagy), and given the important role of autophagy in the oxidative stress response, this warrants further in silico and experimental studies.

4. Conclusions

In the present paper, we demonstrate the potential of using in silico approaches as a starting point to address the complex challenge of understanding the integrated biological roles of microRNAs in oxidative stress responses. By using existing results from published experimental data, combined with databases and software, we predicted a set of 13 common gene targets and 25 commonly affected cellular pathways from a set of 13 miRNAs whose expression levels have been reported to be modulated by oxidative stress. Furthermore, from the 13 identified gene targets, we predicted 21 novel candidate oxidative stress-related miRNAs. Ingenuity pathway analyses of our 13 identified target genes indicated main interaction networks and biological impacts of their gene products and importantly led us to identify protein ubiquitination as a dominating pathway commonly affected by this set of gene targets of oxidative stress-modulated miRNAs. Finally, we found by literature search that we could also draw several lines of connections between our identified novel candidate oxidative stress-related miRNAs and the autophagic pathway.

We consider that our study has two main values. Firstly, the data we have generated can be used as a starting point and resource for the generation of testable hypotheses and further experimental research to address specific questions related to the role of miRNAs in oxidative stress-mediated biological responses. Secondly, the study has value as a proof of principle of how in silico analyses can be used to make advances from already existing data in the field of miRNAs and oxidative stress. Indeed, as the experimental data on oxidative stress-modulated miRNAs, their gene targets, and their biological effects are continuously increasing, there will be more and more to gain by utilizing types of in silico approaches like the ones applied in the present paper. Our study shows that we already have come to the point where such analyses can provide meaningful and useful output, which otherwise would be very hard to reach. We therefore envision an important role for this line of research to be constantly evolving and integrated with biological experimental work, to accelerate our advances in the understanding of the oxidative stress response.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Michele Betti and Maria Cristina Albertini contributed equally to this work.

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Supplementary Materials

Supplementary 1. Table 1: common gene targets of microRNAs with possible roles in oxidative stress. This table is an Excel format of Table 1.

Supplementary 2. Table 2: common gene targets of microRNAs with possible roles in oxidative stress. This table relates to Table 1 and Supplementary Table 1, where gene targets found to be common to 5, 6, or 7 of the 13 oxidative stress-modulated miRNAs were shown. Here, we show the full list of gene targets found to be common to ≥2 of the 13 oxidative stress-modulated miRNAs (i.e., all possible combinations): hsa-let7f (91 elements), hsa-miR-9 (936 elements), hsa-miR-16 (294 elements), hsa-miR-21 (105 elements), hsa-miR-22 (330 elements), hsa-miR-29b (158 elements), hsa-miR-99a (24 elements), hsa-miR-125b (412 elements), hsa-miR-128 (785 elements), hsa-miR-143 (263 elements), hsa-miR-144 (647 elements), hsa-miR-155 (281 elements), and hsa-miR-200c (34 elements). Raw SID1.0 data list is shown. Line numbers indicate code positioning.

Supplementary 3. Table 3: common pathways (KEGG pathway IDs) of microRNAs associated with oxidative stress. This table is an Excel format of Table 2.

Supplementary 4. Table 4: common pathways (KEGG pathway IDs) of microRNAs associated with oxidative stress. This table relates to Table 2 and Supplementary Table 3, where pathways common to all 13 oxidative stress-modulated miRNAs were shown. Here, we show the full list of all KEGG pathways common to ≥2 of the 13 oxidative stress-modulated miRNAs.

Supplementary 5. Table 5: common pathways (KEGG pathway IDs) of microRNAs associated with oxidative stress. This table is an Excel format of Table 3.

Supplementary 6. Table 6: miRNAs predicted to be involved in oxidative stress responses. This table relates to Table 3 and Supplementary Table 5, where miRNAs that target ≥9 of the 13 gene targets of oxidative stress-modulated miRNAs (listed in Table 1) were shown. Here, we show the full list of miRNAs that target ≥2 of the 13 gene targets of oxidative stress-modulated miRNAs (from SID1.0 raw data list).

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Research Article

Oleuropein Aglycone Protects against MAO-A-Induced Autophagy Impairment and Cardiomyocyte Death through Activation of TFEB

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Age-associated diseases such as neurodegenerative and cardiovascular disorders are characterized by increased oxidative stress associated with autophagy dysfunction. Oleuropein aglycone (OA), the main polyphenol found in olive oil, was recently characterized as an autophagy inducer and a promising agent against neurodegeneration. It is presently unknown whether OA can have beneficial effects in a model of cardiac stress characterized by autophagy dysfunction. Here, we explored the effects of OA in cardiomyocytes with overexpression of monoamine oxidase-A (MAO-A). This enzyme, by degrading catecholamine and serotonin, produces hydrogen peroxide (H_2O_2), which causes oxidative stress, autophagic flux blockade, and cell necrosis. We observed that OA treatment counteracted the cytotoxic effects of MAO-A through autophagy activation, as displayed by the increase of autophagic vacuoles and autophagy-specific markers (Beclin1 and LC3-II). Moreover, the decrease in autophagosomes and the increase in autolysosomes, indicative of autophagosome-lysosome fusion, suggested a restoration of the defective autophagic flux. Most interestingly, we found that the ability of OA to confer cardioprotection through autophagy induction involved nuclear translocation and activation of the transcriptional factor EB (TFEB). Our data provide strong evidence of the beneficial effects of OA, suggesting its potential use as a nutraceutical agent against age-related pathologies involving autophagy dysfunction, including cardiovascular diseases.

1. Introduction

The rise in life expectancy has been paralleled by an increased incidence of age-associated diseases such as neurodegenerative disorders and cardiovascular diseases [1]. Heart failure (HF) is a multifactorial clinical syndrome characterized by adverse ventricular remodelling and oxidative stress, which is considered an essential determinant in the progression of ventricular dysfunction. In fact, ROS can directly oxidize proteins involved in contractile activity and consequently impair ventricular function [2, 3]. Although different sources contribute to global oxidative stress, the vast majority of cellular ROS originate from the mitochondrial compartment [4, 5]. In addition to the respiratory chain, a class of enzymes known as monoamine oxidases (MAOs), located at the outer mitochondrial membrane, have been identified as major heart sources of hydrogen peroxide (H_2O_2), a key player in the onset and progression of cardiac injury [6–8]. MAOs are FAD-dependent enzymes found in two isoforms, MAO-A and MAO-B, differing in terms of tissue distribution and

substrate specificity. MAO-A is responsible for the oxidative deamination of catecholamines and serotonin in the heart with production of the corresponding aldehyde, H_2O_2 and ammonia. Although the role of each isoform remains to be investigated, many studies have recently shown that MAO's expression and activity were increased in age-associated chronic cardiac diseases [2, 4, 8, 9].

In the heart, ROS can also interfere with quality control mechanisms by blocking autophagy and promoting senescence and apoptosis [10]. Autophagy is an intracellular process aimed at degrading cytoplasmic components for removal or recycling [11]. At the molecular level, pathological conditions such as HF are characterized by the harmful accumulation of damaged mitochondria and misfolded proteins, probably as a consequence of impairment of the autophagic flux. In such conditions, autophagy is initially activated as a survival mechanism, but under overwhelming stress conditions it can become defective, leading to cell damage [12]. Recent studies have investigated the link between MAO, ROS, and autophagy in HF [8, 13]. Santin et al. [13] reported that MAO-A overactivity was associated with significant mitochondrial dysfunction. Indeed, the MAO-A/H_2O_2 axis negatively affected the elimination and recycling of mitochondria through the autophagosome-lysosome pathway, resulting in cardiomyocyte death and ultimately HF. The authors also reported that, in conditions of enhanced MAO-A activity, the impairment of autophagic flux and lysosomal function were associated with a lack of nuclear translocation of transcriptional factor EB (TFEB), a master regulator of genes involved in the autophagy-lysosomal pathway. Interestingly, TFEB overexpression counteracted the deleterious effects of the MAO-A/H_2O_2 axis by reducing autophagosome accumulation and cell necrosis [13]. Consequently, restoration of defective autophagy now appears as an important therapeutic strategy in the context of cardiovascular diseases [14, 15].

Several small molecules acting as autophagy modulators, such as plant polyphenols, or natural compounds present in fruit and vegetables, have been proposed for their possible therapeutic applications [16–18]. Among these, oleuropein aglycone (OA) is the main phenol present in extra virgin olive oil (EVOO) and is derived from its precursor oleuropein (OLE) by the activity of β-glucosidase released from olive fruits during crushing [19] or present in human intestinal mucosa [20]. The concentration of OA in EVOO ranges from 79 to 229 mg/kg according to Servili et al. [21] and depends on different factors such as olive cultivar, ripening stage at harvesting, and geographic origin of olives. OA has recently been characterized as an autophagy activator and a promising agent against neurodegenerative disorders both in neuroblastoma cell lines and in TgCRND8 mice, a model of Aβ deposition [22–27]. In this transgenic model, food supplementation with OA resulted in remarkable plaque reduction and improvement of cognitive performance by restoring the compromised autophagic flux [27].

Based on the importance of the autophagy process in HF, we sought to assess whether OA-induced autophagy is operative in cardiomyocytes and whether it is protective against cell damage promoted by the MAO/H_2O_2 axis.

2. Materials and Methods

2.1. Materials. Oleuropein was from Extrasynthese (Lyon, France). DMEM (high glucose + GlutaMAX), HAM F-12, fetal bovine serum (FBS), and horse serum were purchased from Gibco, Life Technologies. Medium 199, Earle's salts, pancreatin, gelatin solution, phenol red (solution), Percoll, chloroquine (CQ), and almond β-glycosidase were from Sigma-Aldrich. Collagenase A (*Clostridium hystolyticum*) was from Roche. Ad-MAO-A adenovirus was made as previously described [4]. RFP-GFP-LC3 plasmid was from Addgene (Cambridge, USA).

2.2. Oleuropein Deglycosylation. Oleuropein deglycosylation by β-glucosidase was performed according to Konno et al. [28] with minor modifications. Briefly, a 10 mM solution of oleuropein in 310 μl of 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 8.90 IU of β-(almond) glycosidase overnight at room temperature. The reaction mixture was centrifuged at 36,580g for 10 min to precipitate OA, which was then dissolved in dimethyl sulfoxide (DMSO) with vortexing and sonication. Complete oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma). The mass spectra of oleuropein and of the pellet sample dissolved in DMSO, obtained in ESI and negative ionization mode, by a direct infusion in a triple quadrupole (TSQ Quantum Thermo Finnigan), confirmed the substantial total recovery of OA in the precipitate (Supplemental Figure 1) that, chemically, corresponds to a mixture of isomers (data not shown), also characterized by Diamantakos et al. [29]. The quantity of OA obtained is equimolar to glucose released. A 50 mM OA stock solution in DMSO was stored at −20°C and diluted immediately before use.

2.3. Primary Cardiomyocyte Cultures, Adenoviral Transduction, and Treatment. Isolation of neonatal rat ventricular myocytes was performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. The ventricles were collected from neonatal rats 2–3 days old and were subjected to serial digestions with type II collagenase/pancreatin as previously described [30]. Myocyte enrichment was performed by centrifugation in a discontinuous Percoll gradient, and the resultant suspension of myocytes was plated in the plating medium (68% DMEM + GlutaMAX, 17% Medium 199, 10% horse serum, 5% FBS, and 1.0% antibiotics) onto gelatin-coated culture dishes [13]. The day after the isolation, the medium was replaced with complete fresh medium (HAM F-12, 10% FBS, 10% HS, and 1.0% penicillin/streptomycin).

Adenoviral infection with a replication-deficient adenoviral vector expressing MAO-A was performed as previously described [13]. Ad-MAO-A-transduced cardiomyocytes were either treated with the MAO-A substrate tyramine (TYR, 500 μM) for 6 h to assess the effects of MAO-A activation or pretreated with TYR for 2 h before addition of OA (100 μM) for the remaining 4 h in the culture media (posttreatment).

2.4. SiRNA-TFEB Transfection. Cardiomyocytes were silenced with TFEB siRNA oligonucleotides (SMARTpool ON-TARGETplus, Dharmacon) delivered using the DharmaFECT Duo transfection reagent (Dharmacon). The day after, the cells were transduced with MAO-A adenovirus. At 48 h postsilencing, the cardiomyocytes were stimulated for 6 h with TYR alone (500 μM) or posttreated with OA (100 μM) 2 h after TYR stimulation. Cells transfected with siRNA scramble (Scr) were used as control.

2.5. MTT Assay. The cells were incubated for 3 h in a 0.5 mg/ml MTT solution at 37°C. In the presence of viable cells, MTT is converted into purple formazan crystals insoluble in aqueous solution. Then, the MTT solution was aspirated and 200 μl/well of DMSO was added to solubilize the formazan crystals. Blue formazan absorbance was measured at 570 nm with a spectrophotometric multiplate reader (Bio-Rad).

2.6. Intracellular ROS Generation. ROS generation in cardiomyocytes was measured using the ROS-sensitive DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) fluorescent probe as previously described [13].

2.7. LDH Assay. Lactate dehydrogenase (LDH) released in the culture medium was measured as an index of cell necrosis using the LDH Cytotoxicity Assay Kit (BioVision) according to the manufacturer's instructions.

2.8. Fluorescence Microscopy. Autophagic vacuole staining was performed by the Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences) according to the manufacturer's instructions. Live cells were analysed by fluorescence microscopy, and the fluorescence intensity at 488 nm was quantified using the ImageJ software (RSB). For autophagic flux assessment, plasmid transfection with a GFP-RFP-LC3 construct was performed using the Lipofectamine 2000 reagent (Life Technologies). This construct allows the identification of autophagosomes (GFP^+, RFP^+, yellow) and autolysosomes (GFP^-, RFP^+, red) since the GFP fluorescence is lost upon lysosomal acidification whereas the RFP fluorescence remains stable. The cells were treated according to the experimental protocol, fixed for 5 min in 4.0% formaldehyde solution, washed, and analysed by fluorescence microscopy.

For immunofluorescence studies, the cells were fixed in 4.0% paraformaldehyde, washed again, and incubated with TBS/0.2% Triton for 10 min at room temperature. Then, the cells were blocked with TBS/3.0% BSA for 1 h at room temperature and incubated overnight with anti-TFEB antibody (Bethyl Laboratories) diluted 1 : 400 in blocking solution. The immunoreaction was revealed using Alexa Fluor 546 goat anti-rabbit (diluted 1 : 1000). After washing, the slides were mounted with a cover-slip with mounting medium containing DAPI for nuclei labelling and analysed for TFEB translocation to the nucleus.

2.9. Western Blot. Cardiomyocytes were lysed in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), and 35 μg of proteins was resolved by SDS-PAGE, transferred to a PVDF membrane (by the Trans-Blot Turbo Transfer System, Bio-Rad), and immunoblotted overnight with the following antibodies: anti-LC3B (CST no. 2775, 1 : 1000 dilution) or anti-Beclin1 (CST no. 3495, 1 : 1000 dilution). Then, the blots were incubated for 1 h with specific secondary antibodies (1 : 10000, goat anti-rabbit and goat anti-mouse, Molecular Probes, Life Technologies). β-Actin (Sigma-Aldrich) was used as loading control. Immunoreactive bands were detected by chemiluminescence with a Bio-Rad ChemiDoc XRS^+ camera. Relative densities were quantified using the Image Lab 4.0 software (Bio-Rad).

2.10. Real-Time PCR. Cells were treated with 100 μM OA for 30 min in complete medium. RNA extraction from cardiac ventricles was performed by column affinity purification (Qiagen, Courtaboeuf, France). cDNAs were synthesized using the SuperScript II RT-PCR system (Invitrogen) with random hexamers. Real-time PCR was performed on a StepOnePlus system (Applied Biosystems, Courtaboeuf, France) in 96-well plates with specific primers and a SYBR Green mix (Eurogentec, Angers, France). Primer sequences were as follows: ATP6V1-F: TGTCTCTGGAGTGAATGGTCC; ATP6V1-R: TGCCCACTTCTTTTTGTGCC; Lamp1-F: TGACCATTGTGCTCTGGGAC; Lamp1-R: GGGAAGGTTGATCCTGTGGG; p62 (Sqstm1)-F: CCATCAGAGGATCCCAATGT; and p62 (Sqstm1)-R: CGCCTTCATCCGAGAAAC. Data were normalized using the following primers: GAPDH-F: TCTCTGCTCCTCCCTGTTCTA and GAPDH-R: TCCGATACGGCCAAATCCGTT.

2.11. Statistical Analysis. The results are expressed as mean ± SEM. Experimental groups were compared using Student's *t*-test or the one-way or two-way ANOVA, when appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1. OA Induces Autophagy in Cardiomyocytes. OA has been previously described as a potent and rapid inducer of autophagy in neuroblastoma cells [25]. Thus, we sought to explore whether OA acted in a similar way in cardiomyocytes. Neonatal rat cardiomyocytes were exposed to 100 μM OA, a concentration lacking cell cytotoxicity up to 6 h, the longest incubation time used in our experiments, as verified by MTT and LDH assays (Supplemental Figure 2). At this concentration, OA did not modify baseline oxidative status measured with DCFDA fluorescence (Supplemental Figure 2). Interestingly, after 1 h of treatment with OA, a significant increase in autophagic puncta was evidenced by fluorescence imaging using the Cyto-ID Green dye (Figure 1(a)). Accordingly, we observed that the two autophagy markers Beclin1 (Figure 1(b)) and LC3-II increased at 1 hour following OA stimulation, suggesting early enhancement of autophagy (Figure 1(c)).

3.2. OA Stimulation Enhances Autophagic Flux in Cardiomyocytes. The presence of autophagic vacuoles is not indicative of completed autophagy but can also represent a block in autophagosomal maturation; therefore, we checked the different steps of the autophagic flux in

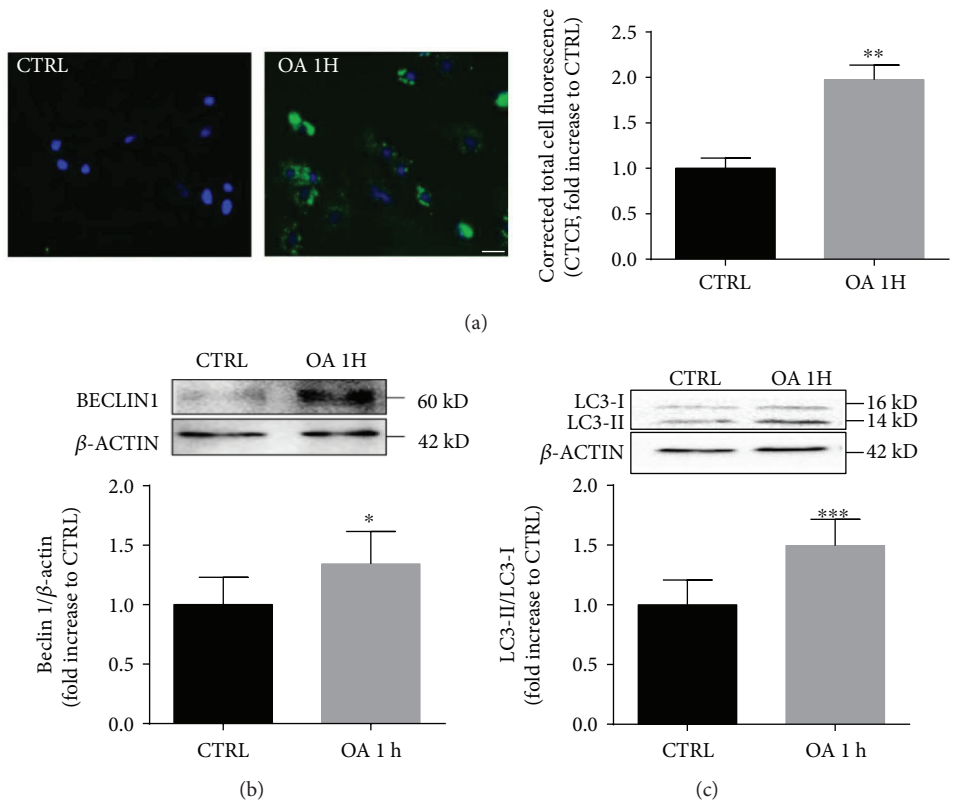

Figure 1: OA induces autophagy in neonatal rat cardiomyocytes. (a) Rat neonatal ventricular myocytes were treated with OA (100 μM) for 1 h, and autophagic vacuoles were stained with Cyto-ID green (left panel). Nuclei were counterstained with Hoechst 33342. Scale bar 20 μm. The graph on the right panel represents quantification of fluorescence intensity ($N = 3$ independent experiments; for each experiment, 6 fields with about 15 cells were quantified per condition). (b, c) Immunoblot analysis of Beclin1 and LC3 proteins was performed on cardiomyocyte protein extracts after stimulation with OA (100 μM) for 1 h. β-Actin expression was used as loading control. The graphs represent quantifications of Beclin1/β-actin ($N = 3$) and LC3-II/LC3-I ratios ($N = 5$) measured by densitometry analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus CTRL (control).

response to OA in cardiomyocytes. To this aim, cells were transfected with the RFP-GFP-LC3 plasmid, resulting in about 30% transfection efficiency, and treated with 100 μM OA for different time periods (1 h, 3 h, or 6 h) and double immunofluorescence imaging was performed (Figure 2(a)). After 1 h of treatment with OA, we observed in the transfected cells an increase in the number of autophagosomes (yellow puncta), which was more pronounced at 3 h. At 6 h, we noticed a decreased number of autophagosomes while autolysosomes (red puncta) increased significantly. This result suggests that OA stimulates autophagic flux in cardiomyocytes. We next confirmed this observation by measuring LC3-II turnover by Western blot. The cells were treated for 6 h with chloroquine (CQ, 10 μM) alone, an inhibitor of lysosome acidification, or with CQ together with OA. As expected, the degradation of LC3-II, the mature form of LC3 incorporated into autophagosomes, was blocked in the presence of CQ, resulting in the accumulation of LC3-II compared to control conditions (Figure 2(b)). In cells treated with both CQ and OA, we found a greater amount of LC3-II compared to CQ alone. This difference might be due to increased autophagosome formation with OA (Figure 2(b)). Taken together, these results indicate that OA is an activator of autophagic flux in cardiomyocytes, including the final steps of autophagosome-lysosome fusion and lysosomal degradation.

3.3. OA Stimulates TFEB Nuclear Translocation and Activity. Once established that OA stimulated autophagic flux in cardiomyocytes, the following step was to elucidate the molecular mechanisms underlying autophagy activation. In this scenario, it has been demonstrated that the transcription factor TFEB acts as a master regulator of autophagy and lysosomal genes, allowing coordination of the different steps of autophagy and activation of the flux [31]. TFEB activity is mainly controlled by its subcellular localization. TFEB is kept inactive in the cytoplasm, but when it translocates to the nucleus, it can activate the transcription of target genes [31]. In order to evaluate the effect of OA on TFEB nuclear translocation, we treated cardiomyocytes for 30 min and visualized TFEB subcellular localization by immunofluorescence staining. As shown in Figure 3(a), in untreated control cells, only 50% of the cells displayed nuclear localization of TFEB while after OA treatment, this percentage increased significantly to 80%. Translocation of TFEB in the nucleus has been demonstrated to allow the transcription of autophagy genes. Thus, we decided to measure the mRNA expression of autophagic genes that are known to be direct targets

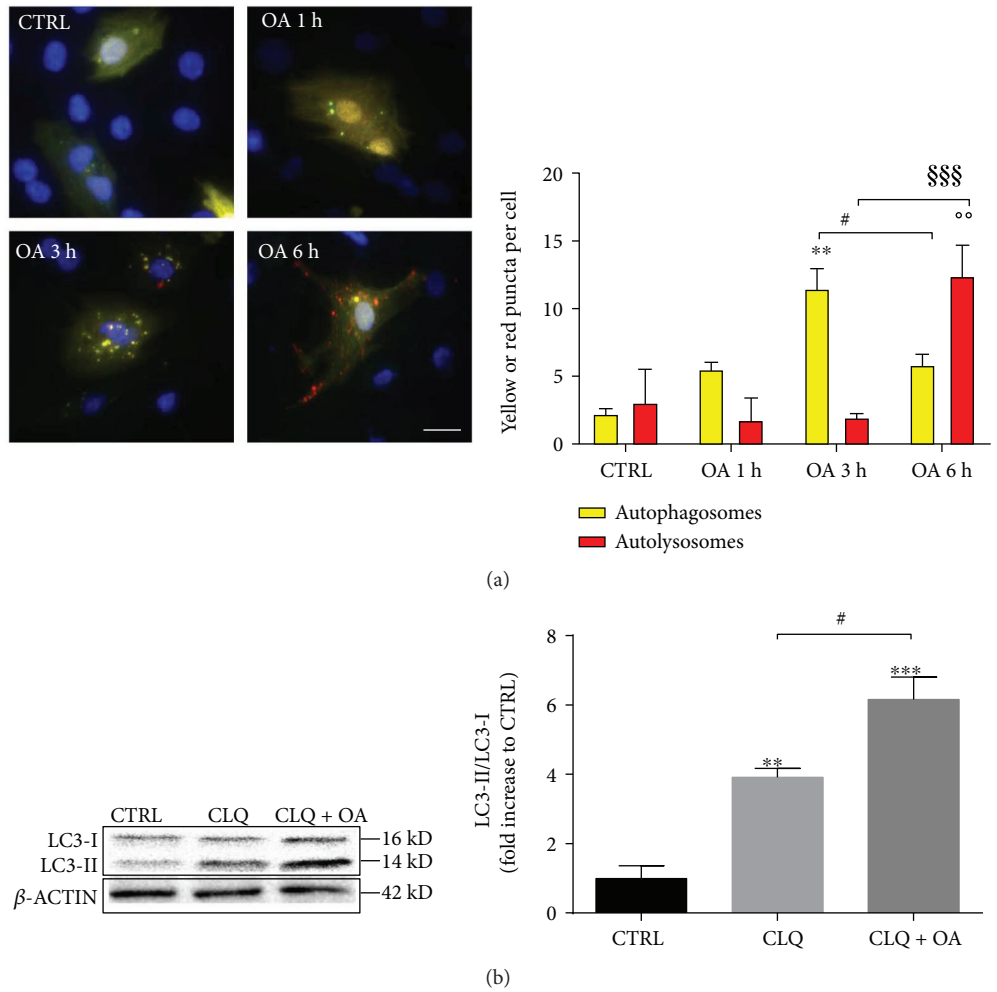

Figure 2: OA enhances autophagic flux in neonatal rat cardiomyocytes. (a) Double immunofluorescence imaging of RFP-GFP-LC3 in cardiomyocytes stimulated with 100 μM OA for different times (1 h, 3 h, and 6 h) (left panel). Scale bar 20 μm. Quantification of yellow puncta (autophagosomes) and red puncta (autolysosomes) for each condition is displayed on the histogram (right panel) ($N = 4$ independent experiments; for each experiment, 10 cells were quantified per condition). $^{**}p < 0.01$ versus CTRL; $^{\circ\circ}p < 0.01$ versus CTRL; $^{\#}p < 0.05$ OA 3 h versus 6 h; $^{\S\S\S}p < 0.001$ OA 3 h versus 6 h. (b) Immunoblot analysis of LC3 protein was performed on cardiomyocyte protein extracts after stimulation with chloroquine (CLQ, 10 μM) or CLQ + OA (CLQ, 10 μM, and OA, 100 μM) for 6 h. β-Actin expression was used as loading control. The graphs represent quantifications of the LC3-II/LC3-I ratio measured by densitometry analysis ($N = 3$). $^{**}p < 0.01$ versus CTRL; $^{***}p < 0.001$ versus CTRL; $^{\#}p < 0.05$ CQ versus CQ + OA.

of TFEB. We found that OA treatment for 30 min increased mRNA expression of Atp6v1, p62, and Lamp1, suggesting early activation of TFEB transcriptional activity (Figure 3(b)).

3.4. OA Protects against MAO-A Induced Cardiotoxicity. Considering our evidence of induction of autophagic flux by OA, we hypothesized that this polyphenol might have beneficial effects in a cardiac model of autophagy dysfunction. In order to evaluate this possibility, we used cardiomyocytes transduced with an adenovirus that drives MAO-A expression (Ad-MAO-A) and we incubated the cells with tyramine (TYR, 500 μM), a substrate metabolized by MAOA; to generate H_2O_2 [13]. In this model, upon TYR addition, we detected a time-dependent increase in ROS production, which was maximal at 1 h, as measured with DCFDA probe fluorescence (Figure 4(a)). As a consequence, mitochondrial dysfunction and cell necrosis were evidenced 2 h after TYR application, as measured with MTT and LDH assays (Figures 4(b) and 4(c)). In order to determine if OA could alleviate the deleterious effects of MAO-A, we first treated the cells with TYR for 2 h and then added OA in the culture media (posttreatment) for the remaining 4 h (Figure 5(a)). Most interestingly, cardiomyocytes subjected to OA posttreatment displayed protection against mitochondrial alteration and necrotic death induced by TYR (Figures 5(b) and 5(c)).

3.5. OA Ameliorates MAO-A-Induced Impairment of the Autophagic Flux. Next, we sought to evaluate whether OA could restore autophagic flux inhibition due to MAO-A activation. To this purpose, we performed the RFP-GFP-LC3

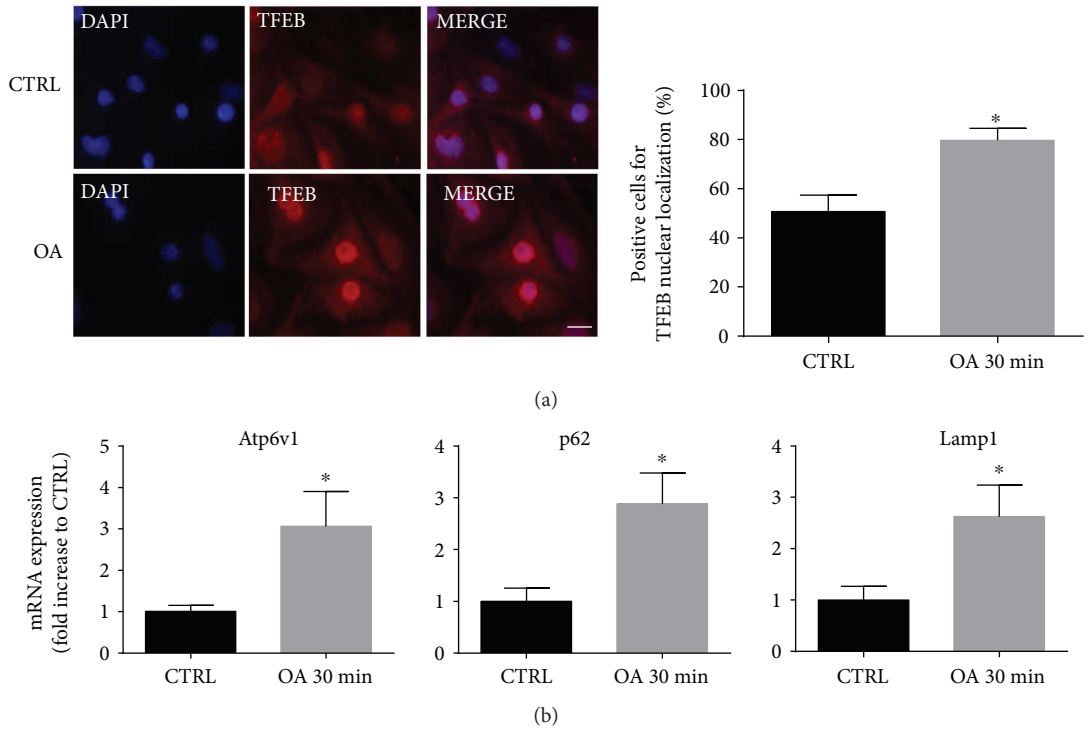

Figure 3: OA induces TFEB translocation and activation. (a) Immunofluorescence staining with TFEB antibody in cardiomyocytes treated with OA (100 μM) for 30 min (red). Nuclei were counterstained with DAPI. Scale bar 20 μm (left side). Quantification analysis of cells with nuclear localization of TFEB in percent of total nuclei (right side) ($N = 3$ independent experiments; for each experiment, 6 fields with about 15 cells were quantified per condition). (b) Real-time PCR expression of Atp6v1, p62, and Lamp1 normalized to GAPDH transcript levels in cardiomyocytes treated with OA (100 μM) ($N = 6$). $^*p < 0.05$ versus CTRL.

assay in cardiomyocytes stimulated for 6 h with TYR alone or with TYR in the presence of OA for the last 4 h (posttreatment). Stimulation of cardiomyocytes with TYR for 6 h induced significant accumulation of yellow puncta (autophagosomes), but not red puncta (autolysosomes), indicative of defective autophagic clearance (Figure 6). However, posttreatment with OA during the last 4 h decreased the number of autophagosomes and increased significantly the formation of autolysosomes in TYR-treated cells (Figure 6). We conclude that OA restores the defective autophagic flux in stress conditions, promoting autophagosome clearance in cardiomyocytes with MAO-A overactivation.

3.6. OA Restores Nuclear Localization of TFEB in Ad-MAO-A-Stimulated Cardiomyocytes. Activation of MAO-A has previously been shown to induce ROS-mediated cytoplasmic build-up of TFEB and reduction of its transcriptional activity, a putative mechanism by which autophagosome clearance was inhibited [13]. We thus sought to explore whether OA might counteract these deleterious effects of MAO-A, by evaluating TFEB subcellular localization with immunofluorescence staining. As expected, TYR stimulation for 2 h significantly decreased the percentage of cells with TFEB nuclear localization compared to untreated (Figure 7). Most interestingly, posttreatment during the last 30 min with OA induced massive translocation of TFEB into the nuclear compartment (Figure 7). These results indicate that the ability of OA to restore the autophagic flux may be dependent, at least in part, on TFEB activation, which is an early event.

3.7. TFEB Activation Is Crucial for OA-Mediated Beneficial Effects. To assess whether TFEB activation was essential for OA protection, we performed TFEB silencing in cardiomyocytes by transfection with TFEB siRNA. We first checked the efficacy of TFEB silencing by quantitative real-time reverse transcriptase-PCR (RT-PCR) and found a significant decrease in TFEB mRNA 24 hours after transfection with TFEB-siRNA, compared to scramble siRNA (SCR-siRNA)-transfected cells (Figure 8(a)). In SCR-siRNA conditions, cardiomyocytes stimulated with TYR for 6 h showed a decrease in MTT, which was also observed in TFEB-siRNA conditions (Figure 8(b)). Interestingly, posttreatment with OA prevented the decrease in MTT in SCR-siRNA conditions, but this beneficial effect was lost in TFEB-siRNA conditions (Figure 8(b)). In conclusion, the presence of TFEB is necessary for OA to confer protection against the deleterious effects of MAO-A.

4. Discussion

In the present study, we report that OA regulates autophagic flux in resting cardiomyocytes and restores autophagy impairment resulting from MAO-dependent oxidative stress, protecting cardiomyocytes from mitochondrial dysfunction and cell death. In addition, to the best of our knowledge, we provide the first evidence of the role

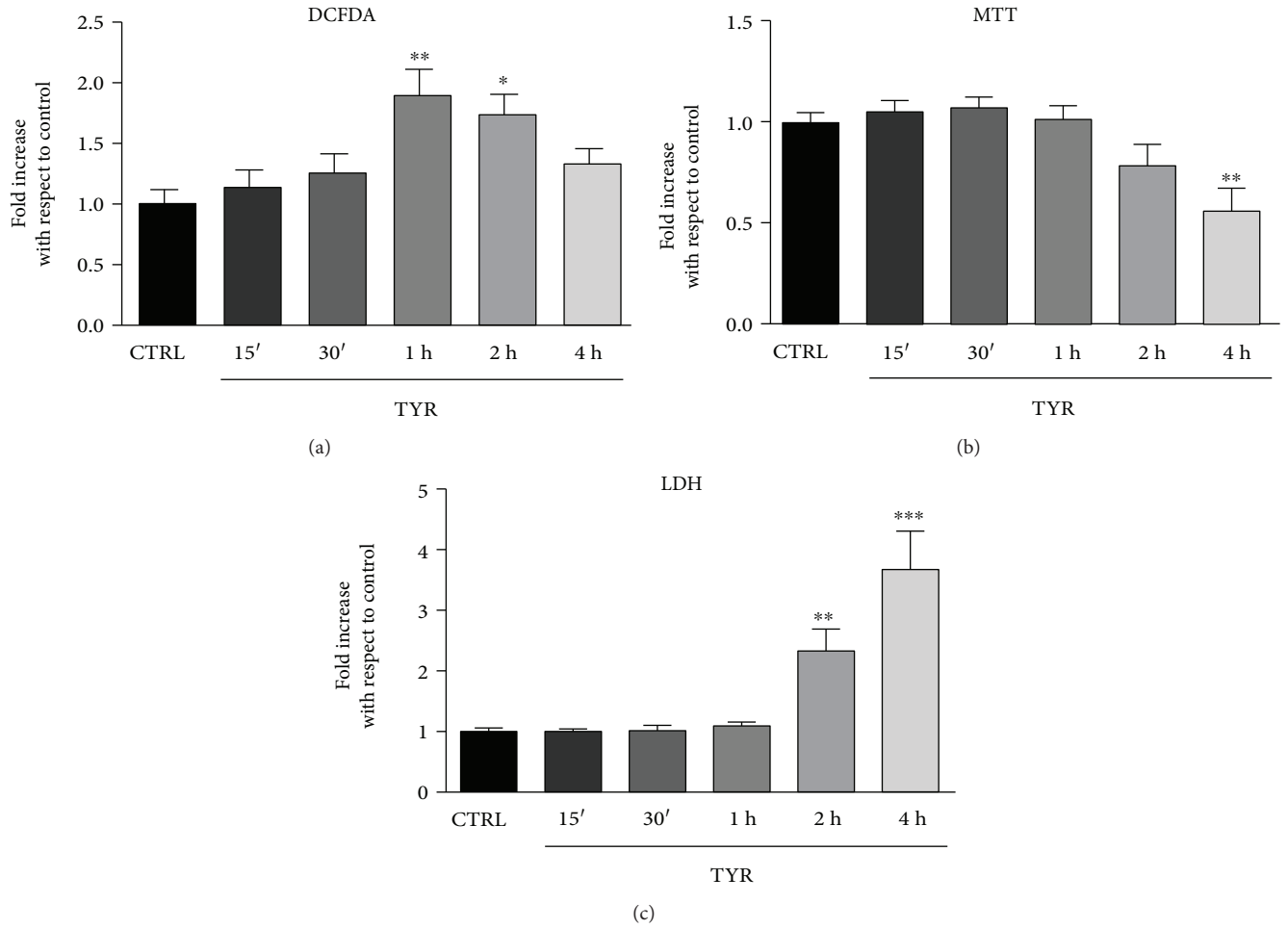

Figure 4: Time-course of DCFDA fluorescence, MTT, and LDH in Ad-MAO-A-transduced cardiomyocytes stimulated with TYR. Ad-MAO-transduced cardiomyocytes were stimulated with MAO substrate TYR (500 μM) for different times (15 min to 4 h) before measuring (a) the fluorescence of DCFDA probe in a fluorimeter ($N = 3$), (b) MTT reduction ($N = 5$), and (c) LDH release in culture supernatant ($N = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus CTRL.

of TFEB activation in the cardioprotective effect of OA against defective autophagy. The cardioprotective effects of OLE and/or OA have been previously attributed to several mechanisms such as reduction of oxidative and nitrosative stress [32] as well as antiplatelet [33], hypolipidemic [34], and anti-inflammatory [35] activities. A recent study [36] shows that OLE reduces proinflammatory cytokines and increases antioxidant markers. Such findings are similar to previous studies showing that OLE reduces prooxidants and proinflammatory cytokines and increases antioxidant markers in adriamycin cardiotoxicity [32, 35] and myocardial ischemia/reperfusion [34, 37]. Another study on acute doxorubicin (DXR)-induced cardiomyopathy suggests that OLE prevents the structural, functional, and histopathological cardiac effects of chronic DXR toxicity, not by a direct antioxidant effect, but through the modulation of signalling pathways of eNOS, iNOS, ET-1, Akt, and AMPK [35].

Autophagy is an evolutionarily conserved self-digestive process through which cells adapt to nutrient starvation and other stress conditions [11, 12, 38]. A major cause of the aging process is a progressive loss of cellular quality control mechanisms, and autophagy is an important quality control pathway, needed to maintain cell homeostasis (notably neuronal and cardiac) and to adapt to stress. A reduction in autophagy has been observed in a number of aging models, and there is compelling evidence that enhanced autophagy delays aging and extends life span. Enhancing autophagy counteracts age-associated accumulation of protein aggregates and damaged organelles [39]. A growing number of studies focus on a causal relationship between impaired autophagic flux and several diseases including neurodegeneration, cancer, myopathy, and cardiovascular diseases [18, 23, 27, 40]. Some data supporting the beneficial effects of plant polyphenols in autophagy-flux impairment were recently reported [41–43]. In our previous studies, we showed that in neuroblastoma cells, OA induces autophagy through activation of the Ca^{2+}/CaMKKβ/AMPK/mTOR signalling pathway, a mechanism in accordance with that previously reported for other polyphenols [24, 25, 27]. *In vivo*, we found that TgCRND8 mice, a model of Aβ deposition, fed with OA-

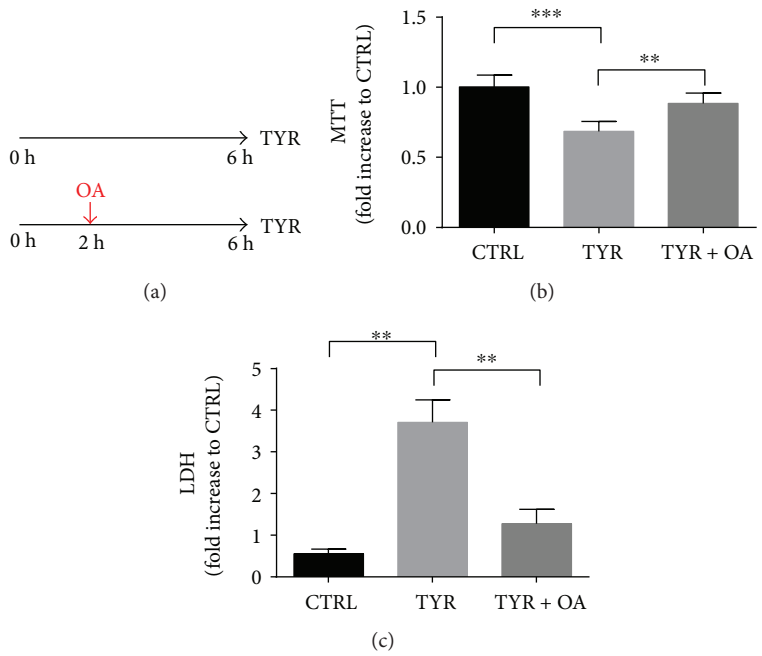

Figure 5: OA is protective against MAO-induced cardiotoxicity. (a) Ad-MAO-transduced cardiomyocytes were stimulated with MAO substrate TYR (500 μM) for 6 h or with TYR + OA during the last 4 h (posttreatment) to measure (b) MTT reduction ($N = 8$) and (c) LDH release in the supernatant ($N = 4$). $^{**}p < 0.01$; $^{***}p < 0.001$ versus CTRL.

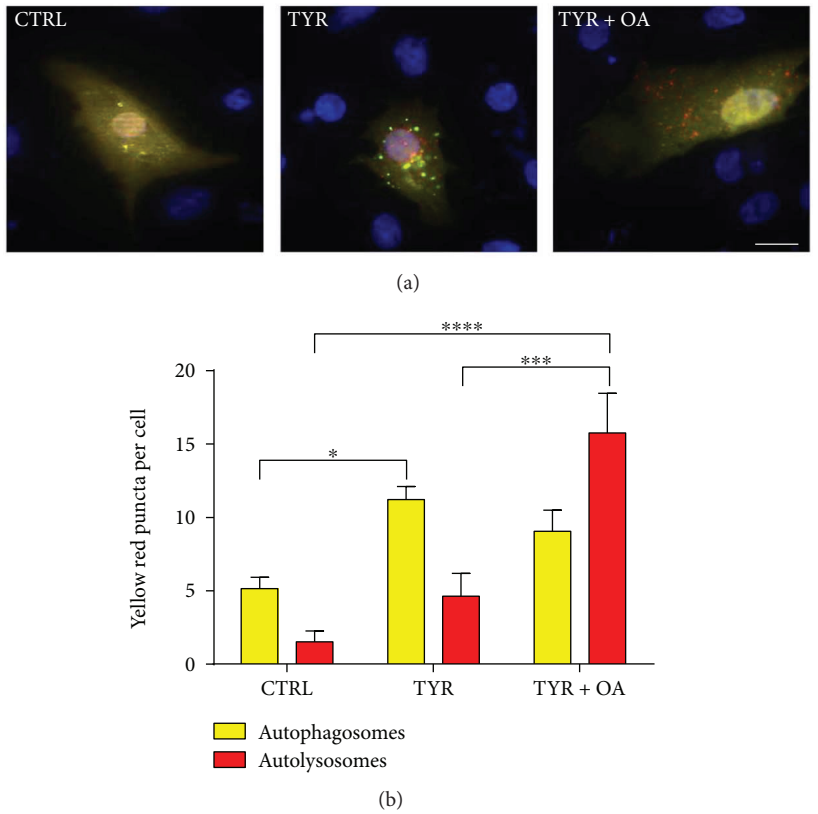

Figure 6: OA restores autophagic flux in Ad-MAO-A-transduced cardiomyocytes. Double immunofluorescence imaging of RFP-GFP-LC3 in Ad-MAO-A-transduced cardiomyocytes stimulated with TYR (500 μM) for 6 h or with TYR + OA added during the last 4 h (posttreatment). Representative images (upper panel). Scale bar 20 μm. Quantification of yellow puncta (autophagosomes) and red puncta (autolysosomes) for each condition is displayed on the histogram (lower panel) ($N = 5$ independent experiments; for each experiment, 10 cells were quantified per condition). $^{****}p < 0.0001$, $^{***}p < 0.001$, and $^{*}p < 0.05$.

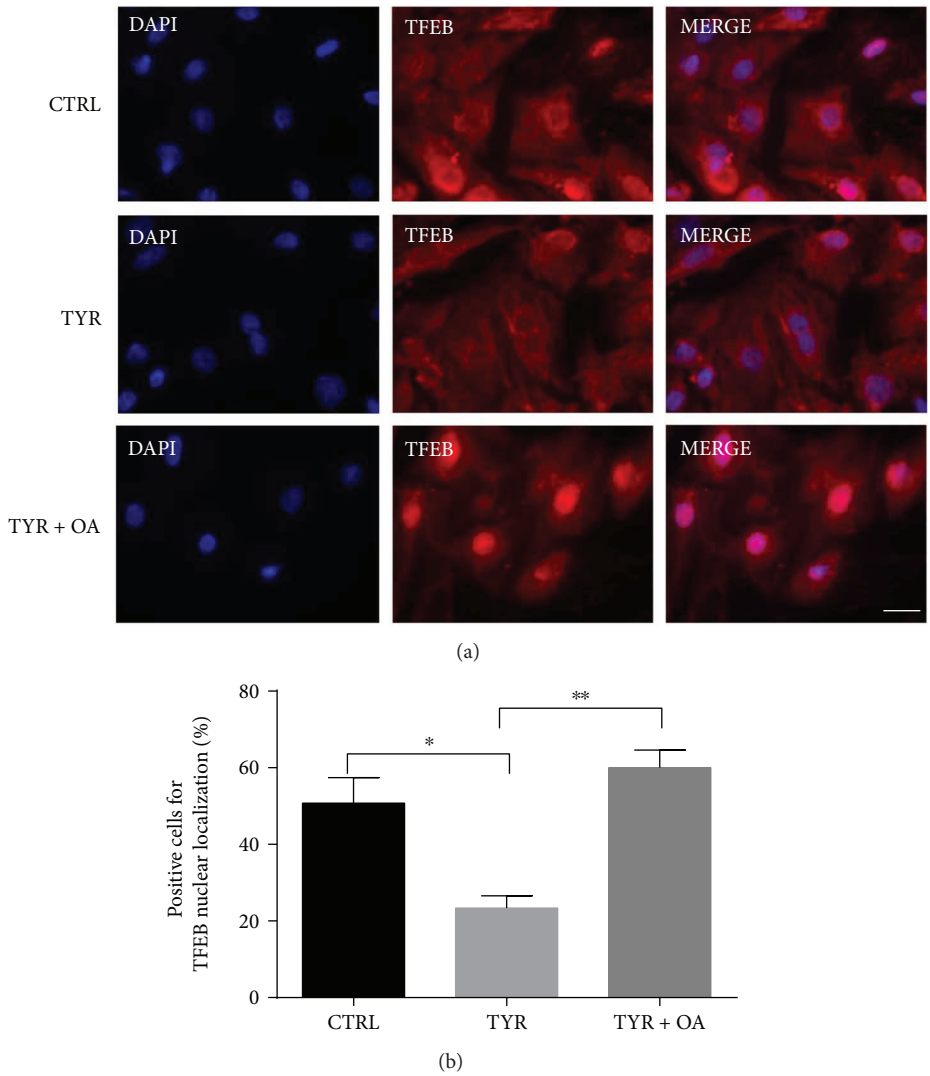

Figure 7: OA induces nuclear localization of TFEB in Ad-MAO-transduced cardiomyocytes. Immunofluorescence staining (red) with TFEB antibody in cardiomyocytes treated with TYR (500 μM) for 2 h or with TYR + OA added during the last 30 min (posttreatment). Nuclei are stained with DAPI (upper panel). Scale bar 20 μm. Quantification analysis of cells with nuclear localization of TFEB in percent of total nuclei (lower panel) ($N = 3$ independent experiments; for each experiment, 6 fields with about 15 cells were quantified per condition). $^{*}p < 0.05$ versus CTRL and $^{**}p < 0.01$ Tyr + OA versus TYR.

supplemented diet displayed a remarkable improvement of the cognitive performance, a massive reduction in Aβ plaque number and size, and an astonishing activation of the autophagic flux in the cortex, where increased expression of autophagy markers was also found [27].

In this study, our first aim was to assess whether OA induced autophagy in cardiomyocytes and, if so, to investigate whether TFEB-mediated transcriptional regulation could play a role. We showed that OA was able to induce autophagy in cardiac cells after short times of treatment, as shown by the increase in autophagic vacuoles and autophagy-specific markers, such as Beclin1 and LC3-II. However, since the accumulation of autophagosomes and the increase of autophagy markers are not fully indicative of effective autophagy activation but can also result from a blockade of autophagosome maturation, we performed an autophagic flux assay. "Autophagic flux" is a measure of degradative completion of autophagy that requires the autophagosome-lysosome fusion and the consequent substrate degradation. Accordingly, complete autophagic degradation is a condition needed to determine whether autophagy is really protective to the cell favouring recycling and cleaning of damaged materials and organelles. To this purpose, in OA-treated cells we used the Tandem Sensor RFP-GFP-LC3B, which specifically labels autophagosomes and autolysosomes, and we found that autophagosomes were processed to lysosomes and that autophagic flux was indeed enhanced. Once having established that OA induced autophagic flux in neonatal rat cardiomyocytes, the next step was to explore the involvement of the master autophagy regulator TFEB. At present, the role of OA as a TFEB activator has not been described. Recent findings indicated that curcumin, another hydrophobic polyphenol, enhances autophagic flux both in human colon cancer HCT116 cells and

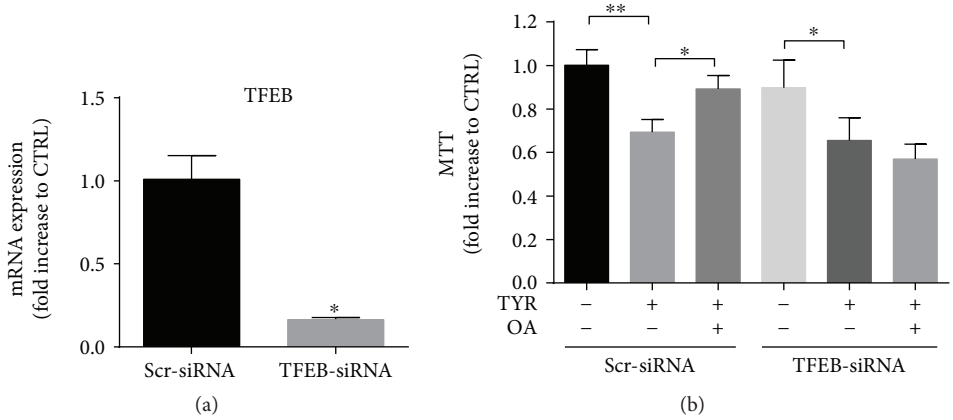

Figure 8: The protective effects of OA on MAO-A-induced toxicity are lost upon TFEB silencing. (a) Cardiomyocytes were silenced with scramble (SCR) or TFEB siRNA, and the level of TFEB mRNA was examined 48 h posttransfection by real time-RT-PCR. Results are normalized to GAPDH and expressed as a fold increase to SCR control siRNA ($N = 3$). (b) Cardiomyocytes were transfected with SCR or TFEB-siRNA for 24 h and then transduced with MAO-A adenovirus for an additional 24 h. Then, cardiomyocytes were stimulated with TYR (500 μM) for 6 h or TYR + OA in the last 4 h, and an MTT test was performed ($N = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$.

in mouse embryonic fibroblasts (MEFs) via mTOR suppression and increased TFEB transcriptional activity [38]. In cardiomyocytes, we found that OA was able to induce nuclear translocation of TFEB and, accordingly, to upregulate TFEB target genes, in particular autophagy genes such as ATP6-V1 ATPase, p62, and LAMP1. TFEB translocation can be regulated by two distinct signalling pathways involving mTOR kinase or calcineurin phosphatase. Under nutrient-rich conditions, TFEB is phosphorylated by mTORC1 on the lysosomal surface, sequestered, and complexed with 14-13-3 proteins. During starvation, mTOR is inhibited, with decreased TFEB phosphorylation and activation of its nuclear translocation. In addition, under starvation, Ca^{2+} released from the lysosome through the MCOLN1 channel activates the calcium-dependent phosphatase calcineurin, which, in turn, dephosphorylates TFEB and induces its nuclear translocation [31]. In the present study, our results show that OA acts as a caloric restriction mimetic, inducing TFEB translocation, eventually through mTOR or calcineurin signalling, which will require further investigation.

Once having highlighted the ability of OA to stimulate autophagic flux through TFEB activation in cardiomyocytes under basal conditions, we sought to better investigate a hypothetical protective effect of OA in stress conditions characterized by autophagy impairment. To this purpose, we used Ad-MAO-A-transduced cardiomyocytes, a cardiac model of autophagy dysfunction characterized by MAO-A overexpression. It is well known that cardiac MAO-A expression increases in rat models of HF, such as hypertension, transverse aortic constriction, diabetes, and cardiac aging [44–46], as well as in human ischemic cardiomyopathy [2]. These pathological conditions are associated with mitochondrial dysfunction and cardiac damage, but only recently, Santin et al. [13] elucidated the reason for the accumulation of dysfunctional mitochondria in situations of enhanced MAO-A activity. MAO-A is responsible for the degradation of serotonin and catecholamines in the heart and produces H_2O_2 as a byproduct of the reaction. Oxidative stress by MAO-A blocks autophagic flux through impairment of lysosomal function, leading to accumulation of damaged mitochondria and to cardiomyocyte necrosis [13]. In addition, MAO-A activation prevents TFEB translocation to the nucleus, reducing its transcriptional activity. We therefore wondered whether the increase in TFEB translocation by OA might mitigate the cardiomyocyte damage caused by the MAO-A/H_2O_2 axis by restoring the autophagic flux. We found for the first time that OA was able to restore autophagic flux in Ad-MAO-transduced cardiomyocytes following massive translocation of TFEB. To exclude the hypothesis that this effect was merely due to an antioxidant effect of OA against MAO-produced H_2O_2, we transfected cardiomyocytes with siRNA targeting TFEB. As transcriptional regulation of autophagy by OA matched with a significant improvement of cell death and mitochondrial functionality that disappeared after TFEB silencing, we hypothesized that TFEB was essential for the protective effects of OA against MAO-A-induced autophagy dysfunction. In addition, in basal condition, OA was able to induce TFEB translocation and autophagy induction without any effect on ROS status in cardiomyocytes, which further highlights its properties as an autophagy inducer.

In conclusion, there is a possibility that TFEB modulation may delay organ degeneration and prevent cardiac disease through restoring functional autophagy. Recently, Sergin et al. [15] were able to reverse autophagy dysfunction of macrophages in atherosclerotic plaques in both tissue and animal models by increasing TFEB function. The authors also showed that a natural sugar, trehalose, induces macrophage autophagy/lysosomal biogenesis, thus recapitulating the atheroprotective properties of TFEB overexpression in macrophages. In line with this, we have shown that the protective effect of OA goes well beyond its known antioxidant power, being able to effectively interfere with key signalling

pathways at the basis of energy metabolism and proteostasis in cardiomyocytes. Our data suggest the possibility to use OA as a nutraceutical in association with the current treatments of cardiovascular diseases characterized by autophagy dysfunction. However, as it has been outlined in a recent review [47], the bioactivity of phenolic compounds is strictly dependent on their bioavailability which represents a critical issue. Concerning OA and its precursor OLE, the main variables are represented by the form in which they are ingested (pure compounds, extracts containing different percentages of the compounds, and whole olive oils with different phenolic composition), dosage, duration of the treatment, and association with different foods. A general consensus concerns that olive oil phenols are absorbed and metabolized by humans, because their degradation and modification products are retrieved in urine following ingestion [48–50]. Nevertheless, absorption profiles vary, depending on the source of such phenols [51, 52]. Moreover, OA is absorbed more efficiently than OLE is, probably because its higher apolarity favours passive transport across the cell membrane [53]. A recent report further shows that, after OLE administration to rats, OA is retrieved both in faeces and in urine (together with hydrolysis and modification products) [54]. Particularly relevant is the evidence suggesting that, in rats and humans, orally administered olive oil phenols, including OA, OLE, and/or one of its derivatives arising from tissue metabolism, are distributed in many tissues, including heart [48, 55]. Finally, OA and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde seem to associate to membranes as a consequence of their hydrophobicity [56]; this implies that they may accumulate at the cellular level, reaching a local concentration higher than that expected on the basis of their plasma concentration.

More information, mainly in human subjects, is still lacking for what OA and OLE effective doses pharmacokinetics and pharmacodynamics are concerned; however, an increasing body of data support the possibility that long-term treatment of aged people with olive leaf-based nutraceuticals and/or EVOO enriched in OA/OLE may contrast the symptoms of aging-related pathologies [57], including cardiac disease, delay their appearance, or reduce their severity.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Jeanne Mialet-Perez and Chiara Nediani contributed equally to this work.

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Supplementary Materials

Supplementary 1. Figure S1: top: oleuropein in DMSO after mass spectra at 539m/z and the [M+Cl−] ion; bottom: the pellet redissolved in DMSO with 377m/z as main ion corresponding to oleuropein aglycone. The absence of ion at 539m/z confirmed the complete hydrolysis of the glycated form.

Supplementary 2. Figure S2: OA is nontoxic in neonatal rat cardiomyocytes. The cells were treated with OA (100 mM) for 6 h, and MTT (mitochondrial functionality), DCFDA (ROS detection), and LDH test (cell necrosis) were performed.

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Research Article

Inhibition of Protein Aggregation by Several Antioxidants

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Amyloid fibril formation is a shared property of all proteins; therefore, model proteins can be used to study this process. We measured protein aggregation of the model amyloid-forming protein stefin B in the presence and absence of several antioxidants. Amyloid fibril formation by stefin B was routinely induced at pH 5 and 10% TFE, at room temperature. The effects of antioxidants NAC, vitamin C, vitamin E, and the three polyphenols resveratrol, quercetin, and curcumin on the kinetics of fibril formation were followed using ThT fluorescence. Concomitantly, the morphology and amount of the aggregates and fibrils were checked by transmission electron microscopy (TEM). The concentration of the antioxidants was varied, and it was observed that different modes of action apply at low or high concentrations relative to the binding constant. In order to obtain more insight into the possible mode of binding, docking of NAC, vitamin C, and all three polyphenols was done to the monomeric form of stefin B.

1. Introduction

Properties of the amyloid state are generic to all proteins [1–3], whereas subtle differences in sequence and 3D structure dictate the propensity to form such ordered aggregates [4, 5]. Even though practically any protein can be induced to form the amyloid state *in vitro*, only about 50 of amyloidogenic proteins aggregate *in vivo* and cause disease [6, 7]. The aggregation of proteins finally leads to amyloid fibrils, which get sequestered into different inclusion bodies, whereas most dangerous proved soluble oligomers, which likely bind and perforate membranes causing toxicity. Therapy for neurodegenerative diseases, which would attack the roots of this pathological process and even cure the disease, is urgently needed, due to the aging population. Ways of how to stop the aberrant process of protein aggregation at a particular point are several [8]. One way is to support the native state by stabilizing antibodies, yet another is to recruit chaperone proteins or augment degradation pathways [9–14]. However, these new ways of possible early treatments are still under investigation in animal models and clinical trials.

Oxidative stress (OS) is one of the most important characteristics of neurodegenerative diseases [15]. It is known that it modifies proteins and causes their misfolding and aggregation. On the other side, protein aggregates bind divalent metal ions (Fe^{2+} and Cu^{2+}), which in combination with hydrogen peroxide leads to the formation of reactive oxygen species (ROS) [16]. Therefore, neuroprotection by reducing protein aggregates or ROS or both seem achievable pharmacological targets [17]. However, very few effective compounds have been developed for clinical application and even fewer have been successful because of their toxicity and potential carcinogenicity. Natural antioxidants provide neuroprotective effects through a variety of biological

actions, such as scavenging free radicals, interaction with transition metals, modulation of different enzymes and effects on intracellular signaling pathways, and gene expression [17]. Several epidemiological studies suggest that diets rich in antioxidants offer protection against numerous pathologies such as cancer, heart disease, hypertension, neurodegenerative diseases, and stroke [17, 18]. One should keep in mind that therapeutic use of relatively safe natural polyphenols is limited by their pharmacokinetics. In addition, these compounds can hardly pass the blood-brain barrier and reach an active concentration in the brain [17]. Some derivatives may act better; for example, it was shown that metal complexes of curcumin inhibited more potently the fibrillation of amyloid-beta (Aβ) than the parent compound did [19].

Knowing that protein aggregation is a shared property of all proteins, model proteins can be used to study this process. We have studied many facets of oligomers and amyloid-like fibril formation by human stefin B [20–25]. Here, we use this system to study the effect of various antioxidant substances on the kinetics, yield, and morphology of amyloid fibril formation. For this study, we have chosen vitamins C (vit C) and E (vit E), *N*-acetyl cysteine (NAC), and three polyphenols: curcumin (Cur), resveratrol (Res), and quercetin (Quer) (Figure 1). Vitamins C and E and NAC, as well as polyphenolic compounds, reduce reactive oxygen species by their free electron scavenging action [26]. However, they also may directly interact with a protein hydrogen-bonding network or aromatic residues, respectively, with an impact on protein aggregation [27, 28].

NAC ((2R)-2-acetamido-3-sulfanyl propanoic acid) (Figure 1(a)) has been shown to be an effective precursor to glutathione (GSH) production, and it is known to cross the blood-brain barrier (BBB) [29]. It provides cysteine, which is the rate-limiting substrate in glutathione synthesis. Therefore, it acts as an antioxidant by increasing GSH levels and by directly interacting with free radicals [29]. Its ability to effectively disrupt the fibrillogenesis of the Aβ peptide has already been reported [30]. Vitamin C ((2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one) is believed to be a vital antioxidant in the brain (Figure 1(b)) [31]. Namely, a huge body of evidence suggests that vitamin C may change the course of neurological diseases and serve as a potential therapeutic tool. Intracellularly, it helps to maintain several key processes, including neuronal maturation and differentiation, myelin formation, synthesis of catecholamine, modulation of neurotransmission, and antioxidant protection [31]. Targeted deletion of the sodium-vitamin C cotransporter in mice resulted in widespread cerebral hemorrhage and death [31].

Polyphenols are secondary plant metabolites characterized by aromatic rings and one or more hydroxyl groups with different structural complexities (Figures 1(c)–1(e)). The most abundant class of phenolic compounds in plants includes flavonoids, such as flavonols, flavones, isoflavones, and anthocyanidins. Resveratrol (3,5,40-trihydroxystilbene) (Figure 1(c)) is an abundant polyphenol, a phytoalexin present in red wine and grapes. It has two phenolic rings connected by a double bond and has two isoforms *trans*-resveratrol and *cis*-resveratrol. *trans*-Resveratrol is believed to be responsible for the French paradox [32, 33]. A huge body of evidence shows its effects on the amyloid fibrillation process [34–37]. One of the most common dietary polyphenols is flavonol quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) (Figure 1(d)). Quercetin is widely present in apples, tea, capers, and onions. Its inhibitory activity toward amyloid fibrillation has already been reported [38–41]. Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) is a yellow pigment present in spice turmeric (lat. *Curcuma longa*) (Figure 1(e)). According to over 6000 citations, it has been associated with many beneficient activities, such as antiamyloidogenic, antioxidant, anti-inflammatory, anticancer, antiviral, and antibacterial [42, 43]. On top of that, over one hundred clinical studies have been carried out with curcumin. Searching the literature, curcumin has been shown to inhibit amyloid fibrillation of fAβ [44, 45], prion protein [46], insulin [47], hen egg white [48], lysozyme [49, 50], and β-lactoglobulin [51]. Unfortunately, its wide application is limited due to its pharmacokinetics, which disfavors its bioavailability. Various formulations of curcumin that are currently available and ongoing studies should help to overcome this problem [42].

2. Materials and Methods

2.1. Materials. In this study, we have used human stefin B (stB wt) as a model protein. This recombinant protein has Cys 3 replaced with Ser. 2,2,2-Trifluoroethanol (TFE) was purchased from Fluka, Thioflavin T (ThT) from Aldrich, and bis(sulfosuccinimidyl)suberate (BS^3) from Thermo Fisher Scientific. Other chemicals were from Sigma, Carlo Erba, Serva, and Merck.

2.2. Expression and Purification. Expression and purification have already been described elsewhere [52]. Briefly, DNA constructs were transformed into the BL21(DE3)-pLysS strain of *E. coli*. Expression was induced with IPTG (final concentration 1 mM). Three hours after induction, cells were separated from the medium and lysed. Expression efficiency was checked by SDS-PAGE electrophoresis. Cell lysates were additionally purified by adding 4% polyethyleneimine (PEI) and repeated centrifugation. This way, most of the contaminants such as nucleic acids and most bacterial (predominantly acidic) proteins were removed from the lysate. The stB wt was isolated from purified cell lysates by affinity chromatography on carboxymethyl (CM)-papain-Sepharose. The nonspecifically bound material was eluted with 0.01 M Tris-HCl containing 0.5 M NaCl at pH 8.0. The wt stefin B was eluted with 0.02 M TEA buffer at pH 10.5. Ionic strength and pH were immediately adjusted with strong 0.2 M phosphate buffer, pH 7, with 1 M NaCl leading to fast refolding. Additional purification was done using SEC on Sephacryl S-200 (Amersham Pharmacia Biotech) equilibrated with 0.01 M phosphate buffer, containing 0.12 M NaCl at pH 6.1. Purity was checked by SDS-PAGE electrophoresis.

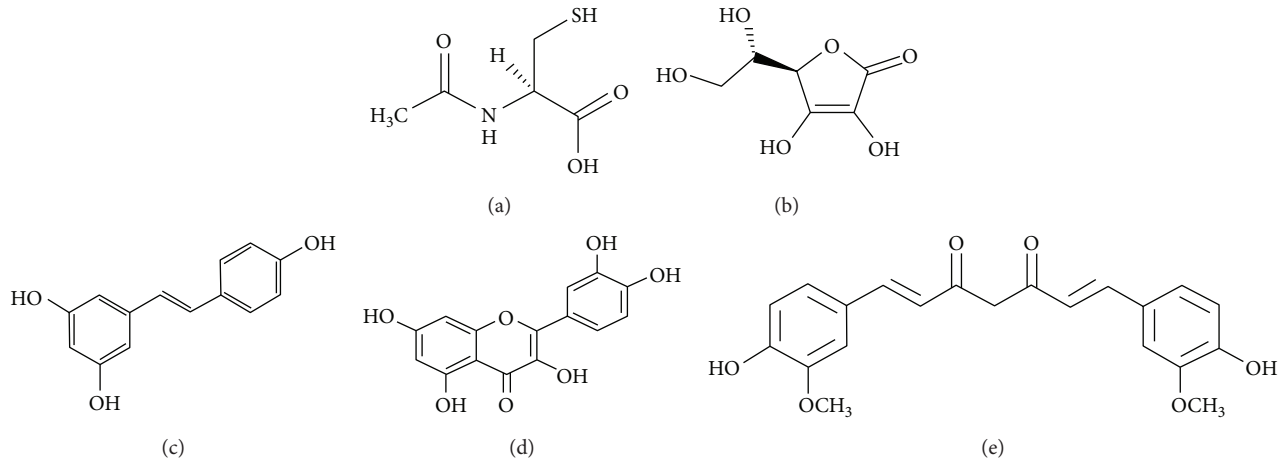

Figure 1: Structures of (a) NAC, (b) vitamin C, (c) resveratrol, (d) quercetin, and (e) curcumin. Computed chemical and physical properties are given in Supplementary Table S2.

2.3. ThT Fluorescence. ThT dye was used to determine the presence of amyloid fibrils. Fluorescence was measured using a PerkinElmer model LS-50B luminescence spectrometer. Excitation was set to 440 nm, and spectra were recorded from 455 nm to 600 nm. ThT dye was dissolved in phosphate buffer (25 mM, 0.1 M NaCl at pH 7.5) at 15 μM ($A_{416} = 0.66$). Fibrils were grown under mild conditions at pH ~5 (0.015 M acetate buffer, 0.15 M NaCl at pH 4.8) at room temperature; protein concentrations were 34 μM. In order to accelerate fibril formation, fibrillation mixtures contained 10% v/v TFE. Fifty microliters of the protein solution in which fibrils were growing was added to 570 μL of the ThT buffer just before measurement. A fresh ThT probe was prepared daily. ThT fluorescence was measured using a 0.5 cm cuvette at 25°C. Excitation and emission slits were set at 5 nm and 7 nm, respectively. Data were collected every 0.5 nm. Fluorescence intensities at 482 nm were plotted against time. Each measurement was performed at least twice in duplicates, and the mean value is presented (average of duplicate measurements ± standard deviations). The blind probe was followed for each antioxidant and subtracted to get final values.

2.4. Transmission Electron Microscopy. Protein samples (15 μL of 34 μM protein solution) were applied on a Formvar- and carbon-coated grid. After 3 min, the sample was soaked away and stained with 1% (w/v) uranyl acetate. Samples were observed with a Philips CM100 (FEI, Netherlands) transmission electron microscope operating at 80 kV. Images were recorded using a Bioscan or ORIUS SC 200 CCD camera (Gatan Inc., Washington, DC, USA), using the DigitalMicrograph software (Gatan Inc., Washington, DC, USA). Two parallel grids were prepared for each sample, at least 10 grid squares were inspected thoroughly, and many micrographs were taken of each grid.

2.5. Molecular Docking. The molecular docking study was performed using the SwissDock server http://www.swissdock.ch/. SwissDock is based on the docking software EADock DSS, whose algorithm consists of many steps [53]. The target molecules were provided as PDB files (stB wt PDB id: 4N6V). Chain A of the protein was selected, water molecules and ions were removed, and all hydrogen atoms were added. A uniform procedure led to numerous predictions for each chosen ligand. Binding modes are scored using FullFitness and clustered. Clusters are then ranked using FullFitness of their elements. In consecutive cycles, the structure of the lowest "FullFitness" and estimated ΔG value was selected and the neighboring docked ligand structures were collected as representative. In other words, only the minimum energy conformation states of the ligand-bound protein complex out of many generated binding modes were considered. Binding modes were visualized in JSmol, and PDB was used for the identification of residues involved in binding.

2.6. ThT Fluorescence Bias Measurement. Fluorescence measurements were done using a TECAN Safire plate reader (Thermo Fisher Scientific) in 96 wells at 25°C. Solutions of stB wt preformed fibrils were incubated at room temperature in the absence and presence of different final concentrations of antioxidants. An 11.4-fold volume excess of ThT was added to each well prior to fluorescence reading. For each assay, the fluorescence of antioxidant without stB wt with ThT dye was also monitored. The excitation wavelength was set at 440 nm, and the emission wavelength was set from 455 to 600 nm. The emission wavelength step size was 1 nm, and excitation and emission bandwidths were set at 7.5 nm. The fluorescence intensity at 482 nm was read. Each sample was followed in triplicate, and the mean value is presented.

2.7. Circular Dichroism. Far-UV circular dichroism (CD) spectra were measured at room temperature by using a Circular Dichroism Spectrometer MOS-500 (Bio-Logic Science Instruments). The fibrillation mixture was prepared as described in Section 2.3. Protein concentration was 34 μM ($A_{280} = 0.15$). The temperature was maintained at 25°C throughout. A 1 mm quartz cuvette was used for all CD spectra. Data were recorded from 250 to 200 nm with a 1 nm sampling interval. The final spectra were the average of three repeated experiments, and the background

(the CD spectrum of the sample without stB wt and antioxidants) was subtracted.

2.8. SDS-PAGE Electrophoresis and Cross-Linking. A fibrillation mixture of stB wt was prepared as described in Section 2.3. Samples aged 24 hours were applied to SDS-PAGE gel using the standard procedure. To make sure that different higher molecular species will remain stable in the presence of SDS and high temperature, we performed cross-linking with BS^3 as a cross-linker. Instructions from the manufacturer were followed, and 50-fold molar excess of the cross-linker has been used. stB wt fibrillation mixture in the absence and presence of the cross-linker was used as control.

2.9. Steady-State Fluorescence Quenching Measurements. Steady-state fluorescence quenching measurements of stB wt in the presence of different concentrations of quenchers, that is, antioxidants, were done using the TECAN Safire plate reader (Thermo Fisher Scientific) in 96 wells at 25°C. The protein concentration was 34 μM, and antioxidant concentrations were varied. Intrinsic tyrosine fluorescence was measured by exciting protein at 277 nm, and emission spectra were recorded in the range of 290 to 360 nm. The excitation and emission slit widths were set at 7.5 nm, whereas emission wavelength step size was set to 1 nm. Each measurement was done in triplicate. The data were analyzed according to the Stern-Volmer equation:

$$\frac{F_o}{F} = K_{sv}[Q] + 1, \quad (1)$$

where F_o and F are the fluorescence intensities in the absence and presence of quenchers, that is, antioxidants, and K_{sv} is the Stern-Volmer quenching constant. Binding constants and binding sites were obtained from (2), which is basically a modified Stern-Volmer equation:

$$\log\left(F_o - \frac{F}{F}\right) = \log K_a + n \log[Q], \quad (2)$$

where K_a is the association constant and n is the number of binding sites [54].

3. Results

The main goal of our study was to assess the effects of different antioxidants on the fibrillation profile of our model protein—human stefin B (stB). Therefore, we have chosen three polyphenols and two compounds with a more simple structure (Figure 1). Firstly, their effects on the amyloid fibrillation profile of stB were followed using ThT fluorescence measurements. It is a usual tool as ThT fluorescence increases in the presence of amyloid fibrils and hence is usually used to characterize inhibitors of amyloid fibrillation reaction. However, precaution is obligatory because ThT fluorescence can be quenched by polyphenol compounds [55–57]. As a complementary tool, TEM images were recorded in the *plateau* phase of the reaction. As described in the Methods section, for each sample, 2 grids were prepared and many images of each were observed to obtain an estimate of the yield of amyloid fibrils. TEM data

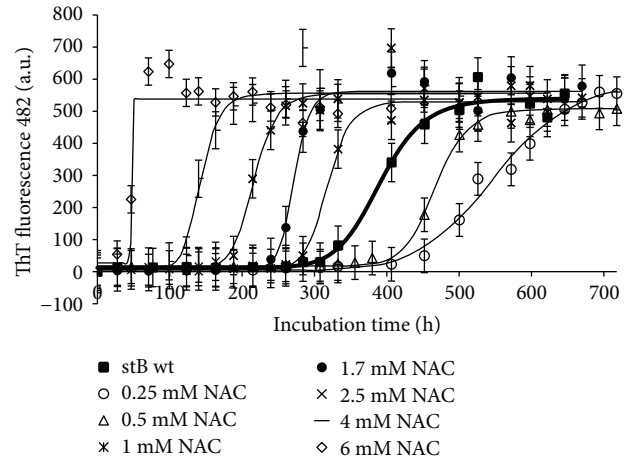

Figure 2: Aggregation kinetics of stefin B monitored by ThT binding/fluorescence in the absence and presence of different concentrations of NAC. ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 μM, and concentrations of NAC were varied. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, prior to mixing with the ThT probe as described in Section 2.

mostly support ThT fluorescence results; however, when quenching was indicated, we measured the ThT fluorescence bias (Supplementary Figure S1). Furthermore, molecular docking was performed in order to obtain predictions of the binding mode for each of the antioxidants. Additional data regarding binding constants were provided using steady-state fluorescence quenching measurements and Stern-Volmer constants. Alterations of the secondary structure were checked using circular dichroism. The oligomeric state was determined using cross-linking prior to SDS-PAGE electrophoresis. Results are described in this section and analyzed in the Discussion section. One part of the results is presented and elaborated in Supplementary materials.

Results of the concentration dependence of NAC and vitamin C on fibril growth as judged by ThT fluorescence are gathered in Figures 2 and 3. In Figure 2, it can be seen that NAC from 1 to 6 mM concentrations (average molar ratio 100 : 1 NAC to protein) promotes protein aggregation, that is, it reduces the lag phase. The final ThT fluorescence intensity does not change substantially (Figure 2); neither does TEM show influence on the final yield or morphology of the mature amyloid fibrils (Figure 4(b) shows 4.0 mM NAC). Lower concentrations, that is, 0.25 mM and 0.5 mM NAC, inhibit fibril growth, that is, prolong the lag phase.

In Figure 3, it can be seen that vitamin C at 2.5 mM and 4 mM concentrations also accelerates the reaction of amyloid fibril formation, that is, shortens the lag phase and lowers the final amount of mature fibrils as judged by ThT fluorescence intensity (Figure 3). This lowering of fluorescence may be partially due to a quenching effect as TEM does not show a lower quantity of mature fibrils at 4 mM or 2.5 mM vitamin C concentrations (Figures 4(c) and 4(d)). At lower concentration, vitamin C acts similarly to NAC; the lag phase is prolonged. Noteworthily, vitamin C at the

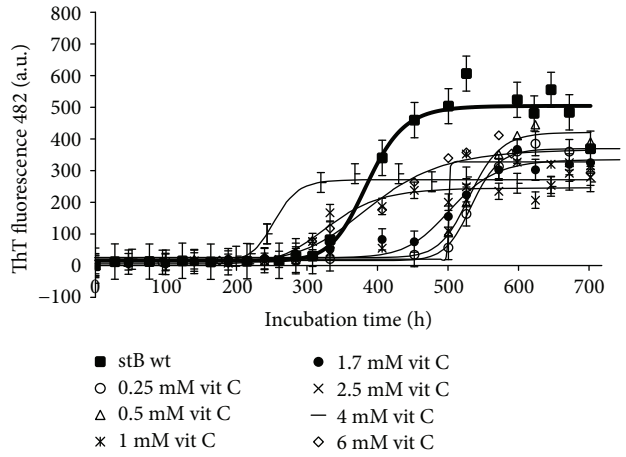

Figure 3: Aggregation kinetics of stefin B monitored by ThT binding/fluorescence in the absence and presence of different concentrations of vitamin C (vit C). ThT fluorescence emission at 482 nm was monitored upon excitation at 440 nm. Protein concentration was 34 μM, and a concentration of vit C was varied. Each sample was incubated at room temperature in 0.015 M acetate buffer, 0.15 M NaCl at pH 4.8, prior to mixing with the ThT probe as described in Section 2.

0.25 mM concentration inhibits fibril growth, that is, prolongs the lag phase (Figure 3) and lowers the mass of fibrils on account of the aggregates as shown by TEM (Figure 4(e)).

We have also measured the effect of vitamin E dissolved in ethanol. No significant effect of this vitamin either on the lag phase or on ThT intensity was observed (data not shown). Fibrils were not inhibited in this case (TEM data not shown).

Results of different concentrations of the three polyphenols resveratrol, quercetin, and curcumin on ThT fluorescence are shown in Figures 5–7. Resveratrol (Figure 5) shows no significant effect on the lag phase, whereas ThT fluorescence intensity slightly decreases in a concentration-dependent manner. However, TEM data (Figures 8(d) and 9(b), F–H) show more aggregates remaining, pointing to a quenching effect (Supplementary Figure S1). Quercetin (Figure 6) prolongs the lag phase; however, this effect is less expressed than for curcumin (Figure 7). Interestingly, when compared to other antioxidants, quercetin shows a different behavior. According to TEM results, more aggregates remain and, in some regions, fibrils look more amorphous (Figure 8(e)). Curcumin affects the lag phase the most (Figure 7), and it also reduces the final ThT fluorescence the most. However, this observation may be due to a strong quenching effect (Supplementary Figure S1). In order to assess the differences in the final quantity of fibrils at certain curcumin concentrations, we have to rely on TEM (Figures 8(f) and 9(b), A–C). This method is not meant to test the amount of the fibrils quantitatively; many grids have to be observed until statistically valid amounts can be estimated. Still, it enables us to estimate the effects of different antioxidants on the final yield and morphology of the aggregates, and the relation between the forms.

We have also measured how the concentration dependence of the three polyphenolic antioxidants, that is, Res, Quer, and Cur, reflects on ThT fluorescence measured at the lag phase of stB fibrillation (Figure 9(a)) as compared to TEM (Figure 9(b)). It can be seen that ThT fluorescence intensity is inversely proportional to the concentration of Res, that is, fluorescence decreases as the concentration of the antioxidant increases (Figure 9(a)). Quer and Cur act similarly—the lowest concentration of both antioxidants did not affect amyloid fibrillation at all or the effect was minor in the case of 1 μM Cur, whereas higher concentrations caused a reduction in fluorescence intensity. However, this effect was more pronounced in the case of Cur (Figure 9(a)). TEM images partially reflect these results (Figure 9(b)). According to TEM results, the final quantity of fibrils in the presence of higher concentrations of Cur (Figure 9(b), C) is lower than in the presence of lower concentrations (Figure 9(b), A). Effects of Quer are specific (Figure 9(b), D and E), and many aggregates are visible in the case of both concentrations. When compared to control, fibrils are shorter and thicker and appear sticky when Cur or Quer is present. TEM data for Res partly support ThT fluorescence results (Figure 9(b), F–H); the inhibitory effect of 100 μM Res is more obvious than that of 50 μM Res. In the case of 200 μM Res, more fibrils are visible.

CD spectra in the far-UV region have not shown any significant change in the secondary structure of stB wt samples aged 24 hours at given concentrations of antioxidants (Supplementary Figure S2). In order to determine the oligomeric state, the fibrillation mixture was subjected to FPLC analysis on Superdex 75 and cross-linking prior to application on the SDS-PAGE gel. The FPLC elution profile could not be used as a reliable result because the protein severely aggregated in the presence of different antioxidants (data not shown). However, SDS-PAGE electrophoresis after cross-linking with BS^3 has shown that antioxidants shift the equilibrium to higher forms, such as tetramers and higher oligomers (Supplementary Figure S3. Tetramers and higher oligomers are marked with an arrow). According to the gel, when protein is exposed to antioxidants, there are more dimers as well, than in the case of the control (Supplementary Figure S3).

A molecular docking study has offered predictions of binding modes for each antioxidant (Figure 10). Thanks to SwissDock, it was possible to obtain docking prediction in JSmol and binding parameters based on the inserted PDB code for stB and the specific structure of the antioxidant. Databank was used to determine amino acid residues that participate in specific protein-ligand interactions (Supplementary Table S1). Interestingly, the program has offered many prediction variants for each antioxidant with the exception of curcumin; this antioxidant has each time been positioned within the same pocket (Figure 10(e), Supplementary Table S1). This observation is supported by results of intrinsic Tyr fluorescence measurements (Supplementary Figures S4E and S5B, D). Supplementary Figure S4E shows a significant decrease in Tyr fluorescence intensity in a concentration-dependent manner, and Stern-Volmer plots and binding constants show that curcumin binds more tightly than do other antioxidants studied here (Supplementary Figure S5B, D).

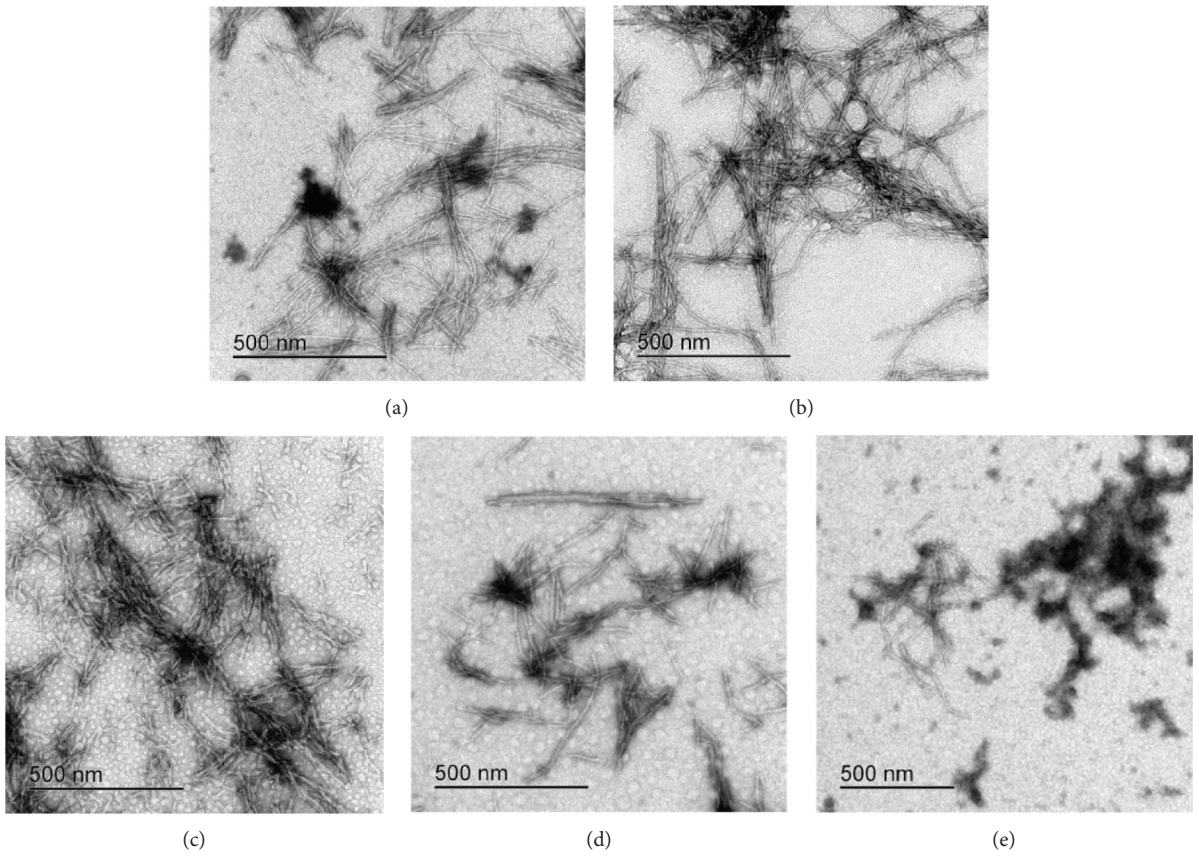

Figure 4: TEM data at the *plateau* of the fibrillation reaction by stefin B. Control: (a) stB in water. Samples: (b) stB with NAC at 4.0 mM and (c) stB with vitamin C at 4.0 mM, (d) 2.5 mM, and (e) 0.25 mM.

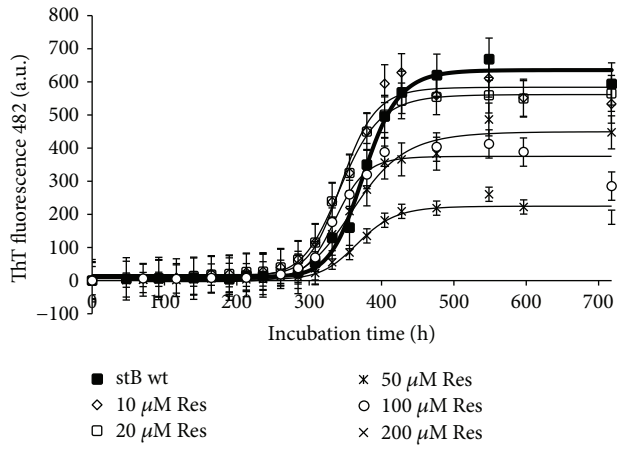

Figure 5: Effect of resveratrol on amyloid fibril formation by stefin B. Aggregation kinetics monitored by ThT binding in the absence and presence of different concentrations of Res. The inhibitory effect on amyloid formation of stB wt was monitored by following the ThT fluorescence emission at 482 nm upon excitation at 440 nm. Concomitantly, the aggregation behavior of stB wt with final 1% v/v DMSO was followed each day as a control because Res was dissolved in DMSO to a final 1% v/v. Protein concentration was 34 μM, and concentrations of Res were varied.

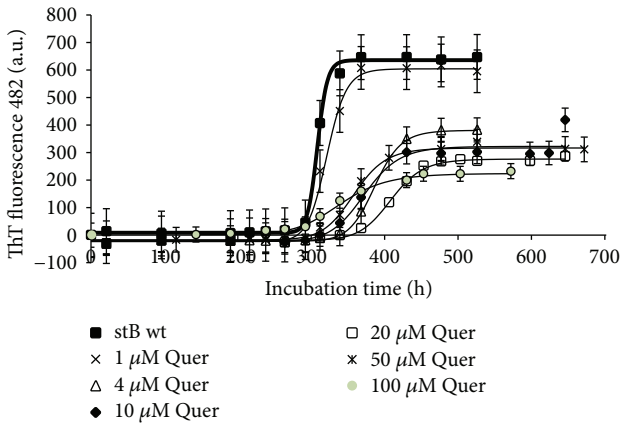

Figure 6: Effect of quercetin on amyloid fibril formation by stefin B. Measurement was done by following ThT fluorescence—as described in Figure 5.

4. Discussion

Protein self-assembly into amyloid fibrillary state is characteristic of numerous debilitating diseases [2]. It is known that natural organic dyes, for example, Congo Red, bind tightly to proteins and hence block their self-assembly. The ability of this and other molecules to prevent amyloid accumulation has generated an immense interest in elucidating their key

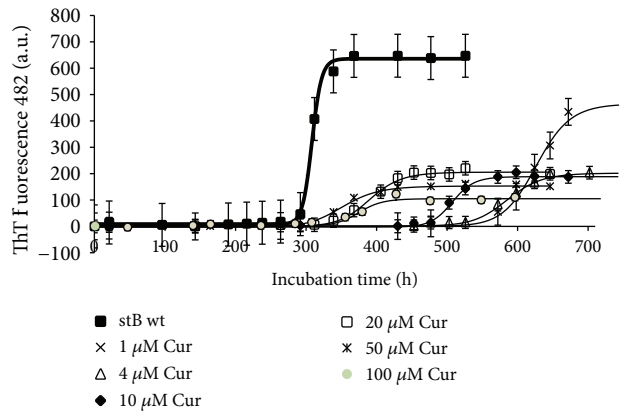

FIGURE 7: Effect of curcumin on amyloid fibril formation by stefin B. Measurement was done by following ThT fluorescence—as described in Figure 5.

structural properties which contribute to inhibitory potency [34, 58, 59]. It is clear that a better understanding of the structure-activity relationships would facilitate the creation of new protein aggregation inhibitors. In turn, these insights might be crucial for deciphering the key elements of the amyloid fibrillation puzzle. Thus, the main goal of this study was to understand at which point the chosen compounds influence protein aggregation reaction and which structural features of the compounds may explain their antiamyloid activity toward our model protein—human stefin B.

The idea that some polyphenolic compounds may interfere with protein aggregation is not new. So far, it is well known that ligands which interfere with amyloid fibrils are flat, planar molecules with substituted aromatic end groups [60]. The first such mention dates back to 2004 when the authors Ono et al. examined the effects of curcumin and rosmarinic acid (RA) on the formation, extension, and destabilization of fAβ(1–40) and fAβ(1–42) [61]. Cur and RA dose-dependently inhibited fAβ formation from Aβ(1–40) and Aβ(1–42), as well as their extension. In addition, they dose-dependently destabilized preformed fAβs [61]. Moreover, in 2006 Porat et al. suggested an additional mechanism of Cur action [28]. By then, the inhibition mechanism of polyphenol antioxidants had been mostly considered as a result of their antioxidative properties. Taking into consideration that polyphenols are capable of inhibiting amyloid fibril formation *in vitro* and in view of their structural similarities, these authors proposed an additional mechanism of action. They suggested that both structural constraints and specific aromatic interactions are important for the inhibition of amyloid fibril formation as they provide proper positioning of the polyphenol inhibitors in the amyloidogenic core.

Our study aimed at showing the effect of various antioxidant substances on the kinetics, yield, and morphology of amyloid fibrils formed by human stefin B *in vitro*. We believe that it mimics other similar systems. To follow the amount of amyloid fibrils, ThT fluorescence measurement was used. It is a usual tool as ThT fluorescence increases in the presence of amyloid fibrils and hence can be used to characterize inhibitors of amyloid fibrillation reaction. However, this assay can be biased by the presence of exogenous compounds, in our case polyphenols, NAC, and vitamin C. There is a study on the interference of the three polyphenols to the intensity of ThT fluorescence [55]. In that study, authors have shown that when it comes to quantification of amyloid fibril formation in the presence of polyphenols, ThT fluorescence should be interpreted with caution. In other words, such compounds can significantly bias ThT fluorescence due to a quenching effect [55]. Therefore, we have checked the quenching properties of each of the chosen antioxidant. Supplementary Figure S1 shows that each compound acts as a quencher of ThT fluorescence. However, results obtained by TEM and the differences in the lag time in ThT fluorescence are still valid to evaluate the inhibitory effects of these compounds on protein aggregation to amyloid fibrils and therefore worth discussing.

ThT fluorescence measurements have not detected any major decrease in the intensity when different concentrations of NAC were added to the stB fibrillation mixture. TEM images similarly did not show any significant difference in the final yield and morphology of stB fibrils (Figure 2 and 4(b)). On the other hand, the lag phase varied in a concentration-dependent manner, that is, higher concentrations have shortened the lag phase, whereas lower concentrations have prolonged it (Figure 2). Noteworthily, a similar behavioral pattern can be observed in the case of vit C (Figure 3), which can probably be explained as their significant influence on the microenvironment of the amyloidogenic core. This observation is not due to pH change because, in our hands, antioxidants did not alter pH. Moreover, electrophoretic techniques can help in determining oligomer sizes in the fibrillation mixture [62]. In our study, SDS-PAGE electrophoresis upon cross-linking has shown that antioxidants NAC and vit C shift the equilibrium to higher forms such as tetramers and higher oligomers (Figure S3; tetramers with a molecular mass of ~44 kDa and higher oligomers are marked with arrows). Similar observations have already been described by other authors [63, 64]. According to the gel, there are more dimers (molecular mass ~22 kDa, marked with an arrow) than in the case of the control (Figure S3). Higher oligomers appear only when protein is exposed to very high concentrations of antioxidants, in our case 2.5 mM and 6 mM vit C (Figure S3, lanes 6 and 7) and 2.5 mM and 6 mM NAC (Figure S3, lanes 9 and 10). Of note, the highest concentrations of both NAC and vit C decreased the lag phase length (Figures 2 and 3).

If we compare our data to the effect of vitamin C to amyloid fibril formation of β-lactoglobulin [27], a similar inhibitory effect is observed. Vitamin C inhibits the fibril formation of stefin B in the range of concentrations from 0.25 mM to 0.5 mM, both by prolonging the lag phase (Figure 3) and by diminishing the final amount of the fibrils, as judged by TEM (Figure 4(e)). Note that ThT may be prone to quenching effects; therefore, one should dissect whether lower fluorescence indeed means a lower amount of the fibrils [55]. Vitamin C at higher concentrations than 0.5 mM (Figure 3) decreases the lag phase and promotes aggregation, similarly to NAC. Final ThT fluorescence appears much lower whereas TEM shows a similar amount

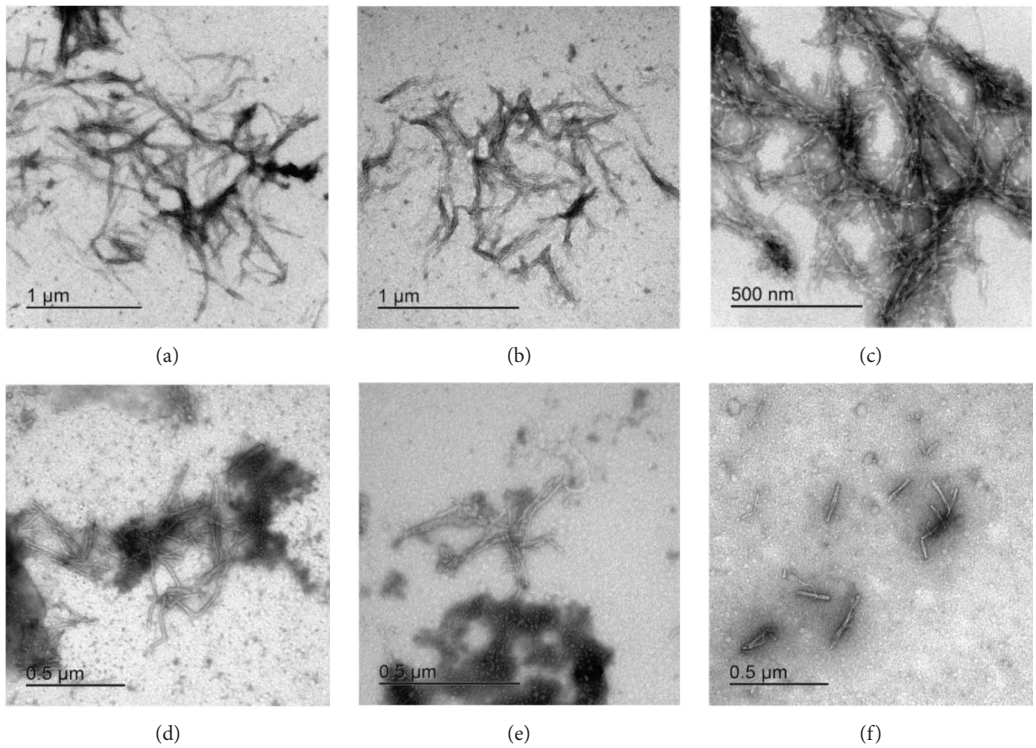

Figure 8: TEM data collected at the *plateau* of the reactions of amyloid fibril formation by stefin B in the presence and absence of polyphenols. Controls (a), (b), and (c) show stB in (a) water, (b) in 1% DMSO, and (c) in 1% ethanol in comparison to 50 μM concentrations of (d) resveratrol, (e) quercetin, and (f) curcumin.

of the fibrils (Figures 4(c) and 4(d)). Here, the discrepancy can best be explained by a severe quenching effect on ThT fluorescence. Lee et al. explained the inhibitory effect of vitamin C through its interference with exposed hydrogen atoms of the N-H groups in the β-sheet backbone [27]. To be specific, metabolites of vitamin C, which are generated in the aqueous solution, such as ascorbate anions and dehydroascorbic acid, can shield electrostatic interactions between β-sheets due to their specific interactions and cause disruption of β-sheet stacking. This mechanism is different from antioxidation, which is usually considered as a major mechanism of vitamin C action. It was even reported that it can decrease amyloid plaque burden in the cortex and hippocampus when tested on cross-bred mice [65]. In our study, it was confirmed once more that vit C has potential as an antiamyloid substance. However, at higher concentrations it may be less beneficial as it increases the most toxic higher oligomers.

The three polyphenols each behave differently. Curcumin at all concentrations inhibited the amyloid fibril formation (Figure 7). Lower concentrations have a greater effect on the prolongation of the lag phase; however, the final mass of the fibrils appears higher. At the concentration of 50 μM, curcumin both prolongs the lag phase and reduces the amount of mature fibrils. The quantity and morphology of the fibrils at 50 μM Cur were checked by TEM and are presented in Figure 8(f). With curcumin, one cannot neglect a quenching effect; therefore, TEM results are more relevant than that using ThT fluorescence. TEM data in Figure 8(f) confirm that curcumin at 50 μM inhibits fibril growth. Noteworthily, the effect follows a concentration-dependent pattern: at lower concentrations, such as 1 μM Cur, the fibrils grow longer, whereas at 50 μM Cur the fibrils are shorter and fewer (Figure 9(b), A–C). Resveratrol does not reduce the lag phase (Figure 5). It apparently reduces the final yield of the fibrils (Figure 5), which however was not confirmed by TEM (Figure 8(d)). Quercetin slightly prolongs the lag phase (Figure 6). However, more aggregates are produced than the fibrils as can be seen by TEM (Figures 8(e) and 9(b), D and E).

Docking studies can provide us with important information and are a very useful tool for understanding the prevailing binding modes between protein and ligands. In order to investigate the possible mode of interaction and determine the most stable complex between stefin B and chosen antioxidants, docking using SwissDock was performed (Figure 10). Docking investigation has offered about 100 different variants for each ligand, and those with the lowest values for free binding energy were chosen (Supplementary Table S1). Interestingly, different positions with different ΔG values were suggested for each ligand except for curcumin, which was each time positioned in a similar way and had the lowest overall ΔG value, as well (Figure 10). When compared to other ligands included in this study, curcumin has a specific chemical scaffold (Figure 1(e)); it contains two substituted aromatic groups symmetrically bound by a short carbohydrate chain. More discussion about why curcumin might be the strongest binder is given in the Supplementary Materials.

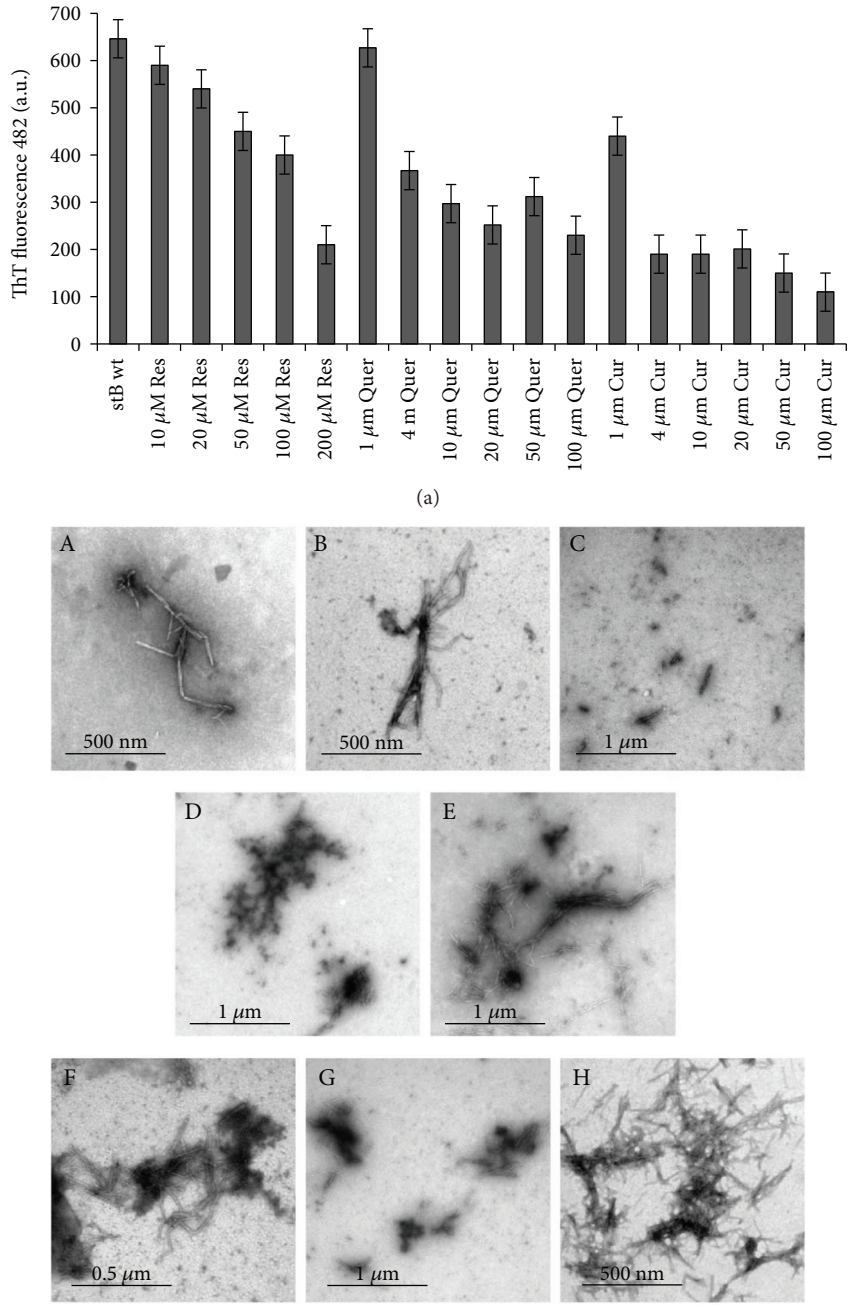

Figure 9: ThT fluorescence intensity versus TEM data. (a) Graph of ThT fluorescence intensity in the *plateau* phase of amyloid fibrillation versus antioxidant concentration. (b) TEM data. Concentration dependence of fibril morphology and approximate amounts; samples were taken in the *plateau* phase of the reactions of amyloid fibril formation by stB at different polyphenol concentrations: (A) 1 μM Cur, (B) 10 μM Cur, (C) 50 μM Cur, (D) 1 μM Quer, (E) 50 μM Quer, (F) 50 μM Res, (G) 100 μM Res, and (H) 200 μM Res.

As a supporting data for docking study, Supplementary Figure S4 shows that each compound also acts as a quencher of tyrosine fluorescence. The observed reduction of fluorescence intensity indicates that each ligand binds in the vicinity of the tyrosine residue—as they come closer to the Tyr residues in the process of binding, their fluorescence intensity is being reduced. Stefin B is a multityrosine protein as it has three tyrosine residues (Tyr53, Tyr85, and Tyr97). Therefore, at first, it might be a complicated task to clarify which tyrosine could be accessible for the ligands. However, this question can be resolved using docking predictions and the human stefin B structure obtained using X-ray diffraction. More information about amino acid residues which participate in binding is given in Supplementary Table S1.

To conclude, in this study, we have studied the effect of 5 different antioxidant compounds on amyloid fibrillation of stefin B. Our results are mostly in line with other

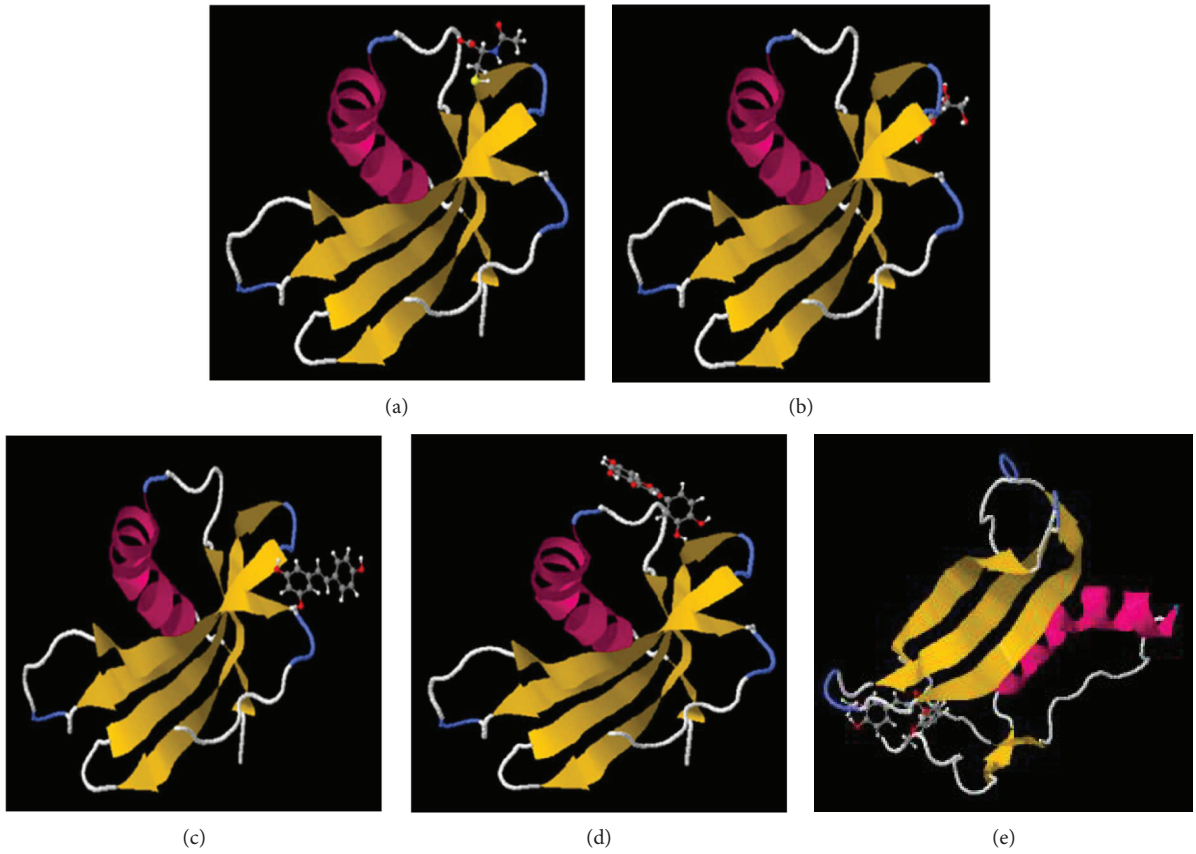

Figure 10: Binding predictions obtained by molecular docking. Molecular docking was performed using SwissDock, and the structures of the most stable stB complexes with (a) NAC, (b) vitamin C, (c) resveratrol, (d) quercetin, and (e) curcumin were selected.

similar studies, showing that antioxidants with flat aromatic structures, as well as vit C and NAC, can interact with the aggregating protein and inhibit amyloid fibril formation at different stages. The current study highlights and partly confirms the possibility that antioxidant compounds can also fight the formation of toxic oligomers and amyloid fibrils. However, their ROS scavenging effect cannot be neglected. Considering that protein misfolding and aggregation cause many debilitating diseases, future studies are warranted.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Samra Hasanbašić and Alma Jahić contributed equally to this work.

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Supplementary Materials

Figure S1: effect of Res, Quer, and Cur and vit C on in situ real-time ThT fluorescence. Figure S2: the CD spectra of stB wt assemblies aged 24 hours in the absence and presence of 50 μM Res. Figure S3: SDS-PAGE of stB wt assemblies aged 24 hours in the absence and presence of different concentrations of antioxidants; samples apart from those in lane 1 were cross-linked before SDS-PAGE. Table S1: molecular docking parameters of the antioxidant-protein interaction. Table S2: computed chemical and physical properties from https://pubchem.ncbi.nlm.nih.gov/. Figure S4: steady-state fluorescence quenching measurements of stB wt in the presence of different concentrations of quenchers, that is, antioxidants. Figure S5: Stern-Volmer plots and modified Stern-Volmer plots of the fluorescence quenching constant for antioxidant-protein complexes. *(Supplementary Materials)*

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Research Article

Low Autophagy (ATG) Gene Expression Is Associated with an Immature AML Blast Cell Phenotype and Can Be Restored during AML Differentiation Therapy

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Autophagy is an intracellular degradation system that ensures a dynamic recycling of a variety of building blocks required for self-renewal, homeostasis, and cell survival under stress. We used primary acute myeloid leukemia (AML) samples and human AML cell lines to investigate the regulatory mechanisms of autophagy and its role in AML differentiation. We found a significantly lower expression of key autophagy- (ATG-) related genes in primary AML as compared to healthy granulocytes, an increased autophagic activity during all-*trans* retinoic acid- (ATRA-) induced neutrophil differentiation, and an impaired AML differentiation upon inhibition of ATG3, ATG4D, and ATG5. Supporting the notion of noncanonical autophagy, we found that ATRA-induced autophagy was Beclin1-independent compared to starvation- or arsenic trioxide- (ATO-) induced autophagy. Furthermore, we identified PU.1 as positive transcriptional regulator of ATG3, ATG4D, and ATG5. Low PU.1 expression in AML may account for low ATG gene expression in this disease. Low expression of the autophagy initiator ULK1 in AML can partially be attributed to high expression of the ULK1-targeting microRNA-106a. Our data clearly suggest that granulocytic AML differentiation relies on noncanonical autophagy pathways and that restoring autophagic activity might be beneficial in differentiation therapies.

1. Introduction

Basal macroautophagy (thereafter referred to as autophagy), a catabolic recycling system in cells, is key to maintaining cellular homeostasis and survival. Furthermore, activation of autophagy allows to extend cell survival when exposed to different types of stressors such as starvation or cytotoxic drugs. The tightly regulated and dynamic process is characterized by *de novo* formation of autophagosomes. Autophagosomes engulf cytoplasmatic components and deliver these cargos, for example, long-lived proteins or damaged mitochondria, to lysosomes for degradation. Studies in yeast have identified a series of autophagy- (ATG-) related genes forming the autophagy machinery. These ATG genes are highly conserved in mammalian cells, allowing to study their functions also in higher eukaryotes [1–4]. Major steps in the autophagic process include initiation, nucleation, elongation, and maturation of the autophagosomes as well as fusion of the autophagosomes to lysosomes. The process of canonical autophagy follows a hierarchical-ordered recruitment of autophagy-related (ATG) proteins to the phagophore assembly site [5]. The autophagy-initiation complex is composed of

ULK1, ATG13, FIP200, and ATG101. The ULK1 protein complex including ULK1, ATG13, and FIP200 coordinates the autophagy initiation from different upstream signaling pathways to induce autophagy [6, 7]. Interestingly, recent data suggest a function for ULK1 not only during autophagy activation but also during elongation and closure of the autophagosomal membrane via binding to ATG8 proteins [8]. Nucleation is under the control of VPS34-Beclin1 class III PI3-kinase complexes resulting in the formation of the isolation membrane [4]. Subsequently, two ubiquitin-like conjugation systems, ATG5/ATG12 and ATG8, the mammalian homologues of which include LC3, GABARAP, and GATE-16, concert the formation of the double-membraned autophagosome [9]. Both systems rely on ATG7, an E1-like enzyme, for activation. Additional proteins involved in these conjugation systems include ATG3, ATG4, ATG10, and ATG16L1. In a last step, autophagosomes fuse with lysosomes to form autolysosomes for the degradation of their contents.

While the importance of autophagy for cell homeostasis and survival has long been appreciated, its role in tumorigenesis and cancer progression is still developing [10, 11]. Autophagy functions in tumor suppression by, for example, preserving protein and organelle homeostasis. Moreover, genome instability was attributed to impaired autophagy and several autophagy genes with tumor suppressor functions (e.g., *BECN1*, *ATG5*, and *ATG4C*) were found [12–14]. On the other hand, cancer cells display increased autophagic activity to meet their increased metabolic needs, and autophagy activated by cytotoxic drugs allows cell survival [15]. Generally, the role of autophagy in tumor development is not fully understood and clearly differs among tumor types and the stage of tumor development. Autophagy may, on the one hand, provide tumor cells with a survival strategy, suggesting a therapeutic use for autophagy inhibition; on the other hand, autophagy may induce cell death by, for example, targeting antiapoptotic proteins, indicating activation of autophagy as novel tool in cancer therapy [16, 17].

Several studies suggested a function for autophagy in mammalian development and cellular differentiation [18–20]. During myelopoiesis, mature myeloid cells undergo a reduction in cell size compared to common myeloid progenitor cells and acquire entirely new morphologies and functions [21, 22]. Such a dramatic change in cell architecture not only implies massive remodeling processes but also requires a delicate balance in macromolecule synthesis and degradation that might be attributed to autophagy. Accordingly, *Atg5* knockout mice showed severe developmental defects [23–25]. In myeloid development, particularly during erythrocyte maturation, the ATG-associated genes *ULK1* (ATG1), *ATG7*, and *NIX* (*BNIPL3*) are critical for the clearance of mitochondria and ribosomes [26, 27]. Furthermore, FIP200, a component of the ULK1 autophagy-initiation complex, is important for hematopoietic stem cell (HSC) maintenance, and *FIP200* knockout HCS displays increased proliferation and myeloid expansion [28]. In general, autophagy is required for HSC survival and the differentiation of adult stem cells including myeloid progenitor cells [6, 20, 27]. Additionally, autophagy is involved in myeloid cell specific functions, such as phagocytosis by monocytes and macrophages [29, 30] as well as antigen presentation by dendritic cells [31]. Lastly, autophagy deficiency led to defects in neutrophil degranulation and reduced the inflammatory potentials of neutrophils [32].

Acute myeloid leukemia (AML) is an aging-related, genetically highly heterogeneous blood cancer subtype that is characterized by the accumulation of myeloid blast cells with altered self-renewal, proliferation, and differentiation function [33]. Acute promyelocytic leukemia (APL), a particular AML subtype, is characterized by the translocation t(15,17) encoding for the oncogene-retinoic acid receptor alpha (PML-RARA) fusion protein [34]. PML-RARA prevents effective transcription of RARA target genes important for myeloid differentiation in a dominant negative manner. Moreover, PML-RARA represses transcription of PU.1 transcriptional targets by binding to overlapping DNA binding sites. Since PU.1 controls transcription of a series of myeloid genes, its inhibition by PML-RARA contributes to the impaired differentiation seen in APL [35]. All-*trans* retinoic acid (ATRA) in combination with anthracyclines or arsenic trioxide (ATO) is able to induce complete remission in 90% of the patients by inducing PML-RARA degradation via the proteasome or caspase cleavage [36, 37]. In addition, ATRA induces Beclin1-independent autophagy or aggrephagy that contributes to the degradation of PML-RARA protein aggregates [38–40]. Furthermore, we and others reported that ATRA-mediated AML differentiation depends on active autophagic flux and that inhibition of autophagy by pharmacological and genetic means attenuated ATRA-induced neutrophil differentiation of APL and non-APL cell lines [40–42]. This also indicates that autophagy is involved in myeloid differentiation beyond its role in the degradation of PML-RARA aggregates [38].

Despite several studies analyzing the function of autophagy during myeloid differentiation [43–48], the myeloid autophagy pathway active during this process is not yet fully characterized, and clinical data on general ATG expression in primary AML are rare. In this study, we show that ATG expression is frequently repressed in primary AML patients and that neutrophil differentiation of AML cells depends on functional autophagy distinct from the canonical pathway. We identified several ATG genes as novel transcriptional targets of PU.1 and speculate that low ATG gene expression in AML is partially due to low PU.1 levels. Lastly, AML differentiation causes downregulation of microRNA-106a, allowing for the expression of its target ULK1. Accordingly, preliminary data indicate that low ULK1 expression is associated with increased miR-106a expression in a small cohort of AML patient samples.

2. Results

2.1. ATRA-Mediated Cellular Differentiation of AML (HL60) and APL (NB4) Cells Is Associated with Increased, Noncanonical Myeloid Autophagy. To date, activation of autophagic flux during ATRA-induced APL/AML differentiation has mostly been studied during short time exposure to ATRA and was not directly compared to starvation or arsenic

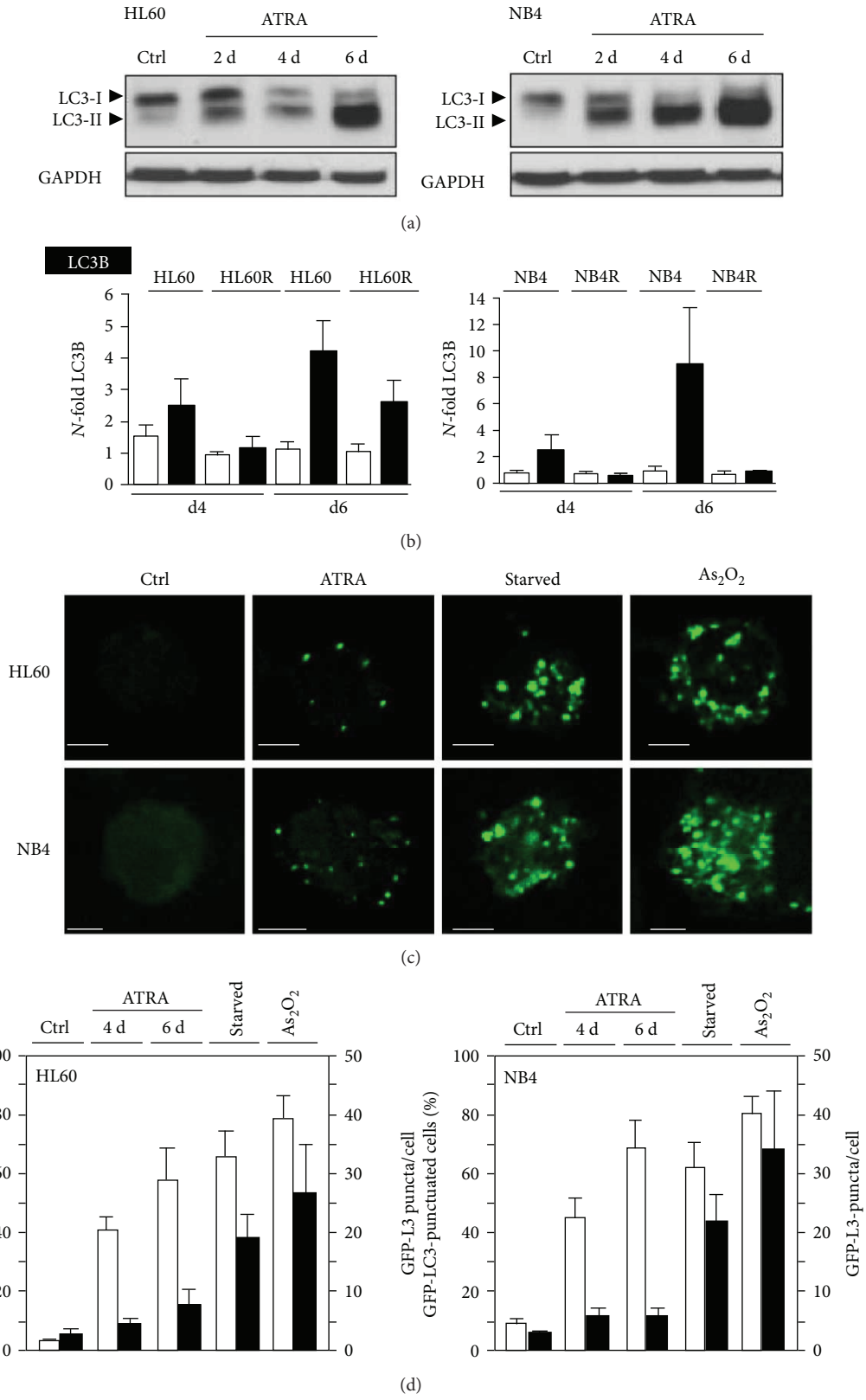
trioxide- (ATO-) induced autophagy [38]. Thus, we investigated in more detail how ATRA activates autophagy in non-APL HL60 as well as in APL NB4 cells. We found a marked induction of autophagy during neutrophil differentiation starting from day 2 in both cell lines as indicated by a marked shift from LC3B-I to LC3B-II (Figure 1(a)). As a second autophagy marker, LC3B mRNA induction was analyzed upon ATRA treatment in HL60 and NB4 as well as in their respective ATRA-resistant subclones. LC3B mRNA was significantly upregulated in both parental cell lines upon ATRA treatment but not in the resistant sublines, except for a marked but less pronounced increase at day 6 in the ATRA-resistant HL60 line (Figure 1(b)). Additionally, GFP-LC3B redistributed from a diffuse pattern to a punctuate pattern in HL60 and NB4 cells upon neutrophil differentiation, further indicating active autophagy (Figure 1(c)). Interestingly, quantification of GFP-LC3 dots revealed that starvation- and ATO-induced autophagy in the same cells was clearly different from ATRA-mediated induction of autophagy (Figure 1(d), black bars). Still, the percentage of cells with GFP-LC3B puncta was similar for all three treatments (Figure 1(d), white bars). Lastly, to confirm induction of autophagy flux upon ATRA treatment, we generated NB4 cells expressing the mCherry-EGFP-LC3B tandem construct. Autolysosomes appear red since the lower pH in these organelles quenches the EGFP signal. The ratio of mCherry to EGFP fluorescence was determined by flow cytometry as described earlier [49]. A threshold was set to identify the percentage of cells with high autophagic activity (high mCherry/EGFP ratio). We found a shift towards red fluorescence in NB4 cells treated with ATRA (Figure 1(e), left panel) together with a significant increase of cells with high autophagic activity (Figure 1(e), right panel).

To test whether autophagy is essential for neutrophil differentiation of HL60 AML cells, we blocked autophagy pharmacologically using the phosphatidylinositol 3-kinase (PI3K) inhibitor 3-methyladenine (3-MA) or chloroquine (CQ). Inhibition of ATRA-induced autophagy resulted in diminished neutrophil differentiation of these cells as evidenced by significantly reduced CD11b surface expression (Supplementary Figure 1a). In order to exclude that inhibiting autophagy influenced neutrophil differentiation solely by increasing apoptosis, we cotreated these cells with the pan-caspase inhibitor z-VAD-fmk. Blocking apoptosis during 3-MA or CQ-mediated inhibition of ATRA-induced autophagy did not show any effects on neutrophil differentiation (Supplementary Figures 1a and b, and data not shown). Our data clearly suggest that induction of autophagy is a prerequisite for ATRA-induced neutrophil differentiation of AML cells per se whereas its role in ATRA-induced cell death is negligible.

2.2. ATG Gene Expression Is Frequently Reduced in Primary AML Patients. We previously published that ATG genes such as *DRAM1*, *WIPI1/2*, and *MAP1S* were significantly downregulated in primary AML samples [42, 50, 51]. Nevertheless, a more global ATG gene expression profile in clinical AML patient samples is still missing. Therefore, we quantified the expression levels of additional 18 ATG genes in a cohort of 114 AML patient samples with defined chromosomal aberrations and compared them to the corresponding expression levels in mature granulocytes from healthy donors ($n = 13$) (Figures 2(a)–2(d) and Supplementary Figure 2). The expression of 9/18 ATG genes, operative in different phases of autophagosome biosynthesis, was significantly inhibited in AML as compared to their expression in mature, healthy granulocytes: *ULK1, FIP200, BECN1, ATG14, ATG5, ATG7, ATG3, ATG4B, and ATG4D*. Our data suggest that low ATG gene expression is associated with an immature AML blast phenotype.

2.3. Granulocytic Differentiation of AML/APL Cells, Primary Human APL Cells, and Healthy Human CD34$^+$ Progenitor Cells Is Paralleled by Increased ATG Gene Expression. Based on our findings in clinical AML samples showing globally reduced ATG gene transcription compared to mature granulocytes, we asked if these genes are induced during leukemic and normal neutrophil differentiation. To this end, we first analyzed expression of selected ATG genes, ATG3, ATG4D, and ATG5 during neutrophil differentiation in APL cell lines as well as in primary APL patients receiving ATRA therapy. Due to limited patient sample RNA, we could only determine *ATG5* expression in APL patients. We found that all three ATG genes investigated were significantly induced upon 4 days of ATRA treatment in parental but not in ATRA-resistant NB4 cells (NB4-R2) (Figure 3(a)). Importantly, marked *ATG5* induction was seen in primary APL patients upon ATRA therapy *in vivo* during short- and long-term follow-up examinations (Figure 3(b)). Lastly, to test if ATG gene expression is also induced during normal granulocytic differentiation, we differentiated human CD34$^+$ progenitor cells towards neutrophils using G-CSF. Similar to leukemic neutrophil differentiation, all three ATG genes analyzed showed a significant increase in their expression levels (Figure 3(c)). Together, neutrophil differentiation is positively correlated with increased expression of key ATG genes.

2.4. Inhibiting the Key ATG Gene ATG5 Significantly Attenuates Granulocytic Differentiation and Autophagy of AML Cells. To test if ATRA-induced autophagy depends on the conserved elongation process during autophagy, we knocked down *ATG5* in two AML differentiation models. We first analyzed ATG5 protein expression during neutrophil differentiation of HL60 AML and NB4 APL cells. We found a time-dependent upregulation of ATG5 protein levels in both cell lines during ATRA differentiation (Figure 4(a), left panels). To exclude that ATG5 induction is solely a consequence of a stress response to ATRA treatment rather than being functional in myeloid differentiation, we analyzed ATG5 expression in ATRA-resistant HL60-R and NB4-R2 sublines. Both cell lines did not upregulate ATG5 upon ATRA treatment compared to the parental cells (Figure 4(a), right panels), further suggesting that upregulation of ATG5 is associated with neutrophil differentiation. Importantly, knocking down *ATG5* using two independent shRNAs significantly reduced ATRA-induced autophagy (Figures 4(b) and 4(c)). In parallel with disabling ATRA-induced autophagy, AML *ATG5* knockdown cells showed



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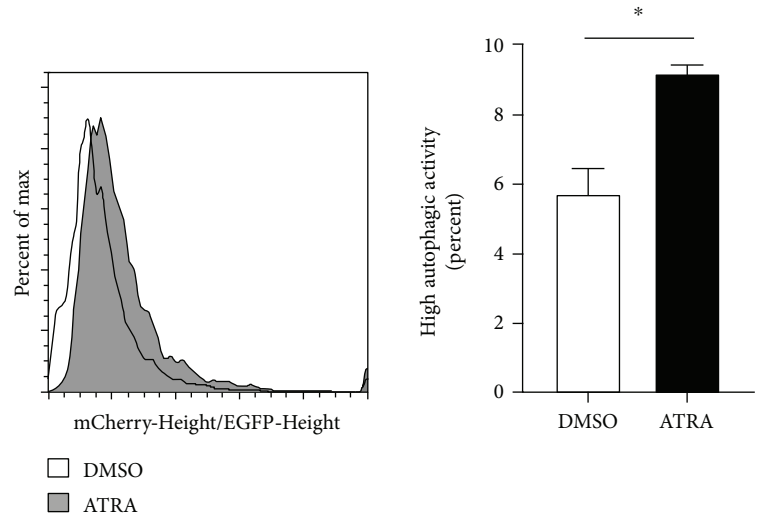


(e)

Figure 1: Autophagy is associated with neutrophil differentiation of AML cells. (a) ATRA differentiation induces LC3B lipidation measured by Western blotting. HL60 AML and NB4 APL cells were treated with 1 μM ATRA for up to 6 days. GAPDH was used as a loading control. (b) qPCR analysis of LC3B mRNA of HL60 and NB4 as well as ATRA-resistant HL60-R and NB4-R2 cells after 4 and 6 days of ATRA treatment, respectively. Data are shown as N-fold activation compared to untreated cells at days 4 and 6. (c) ATRA differentiation induces GFP-LC3 dot formation. NB4 and HL60 cells stably expressing GFP-LC3 were treated with 1 μM ATRA, starved for 8 hours, or treated with 6 (NB4) and 12 μM (HL60) arsenic trioxide (As_2O_3), respectively. GFP-LC3 puncta were detected using confocal microscopy. Scale bar 10 μm. (d) Treatment as in (c). Results are expressed as percentage of cells showing punctuated GFP-LC3 staining and as average number of puncta per cell. Counts are mean ± s.e.m.; $n = 100$; three independent experiments. (e) FACS analysis of NB4 cells expressing the tandem construct mCherry-EGFP-LC3B. Left panel: histogram of the mCherry-Height/EGFP-Height ratio in cells treated with vehicle or with ATRA (1 μM) for 48 h. Right panel: percentages represent cells with high autophagic activity based on a threshold set on control cells. Mann–Whitney U test, $^*p < 0.05$.

impaired neutrophil differentiation (Figure 4(d)). Moreover, inhibition of apoptosis using caspase inhibitors had no effect on reduced neutrophil differentiation upon ATG5 inhibition (Figures 4(d) and 4(e)), suggesting that reduced cellular differentiation is not due to increased cell death of *ATG5* knockdown cells. Importantly, combining ATRA with the pharmacological autophagy inducers everolimus or LiCl resulted in significantly enhanced neutrophil differentiation as assessed by elevated CD11b surface expression paralleled by increased ATG5-ATG12 complex formation (Figure 4(f)).

To further validate earlier findings regarding the type of autophagy activated by ATRA, we knocked down *Beclin1* in HL60 AML and NB4 APL cells. As described above, we found significantly lower *Beclin1* levels in primary AML samples as compared to mature neutrophils. In line with these data, we observed slightly increased Beclin1 protein upon ATRA-induced neutrophil differentiation of both AML cell lines (Supplementary Figure 3a). Importantly, knocking down *Beclin1* did not abrogate ATRA-induced autophagy (Supplementary Figures 3b and c, left panels). At the same time, inhibition of Beclin1 significantly reduced starvation- and arsenic trioxide- (ATO-) induced autophagy in HL60 and NB4 cells, confirming the functionality of the *Beclin1* knockdown (Supplementary Figure 3c, right panels). In line with its negligible role in ATRA-induced autophagy, *Beclin1* knockdown AML cells displayed no significant reduction in neutrophil differentiation (Supplementary Figure 3d). Thus, ATRA-induced autophagy seems clearly different—less intense and Beclin1-independent—from canonical starvation- or ATO-induced autophagy.

2.5. Transcriptional Regulation of Key ATG Genes by the Myeloid Master Regulator PU.1. The Ets family transcription factor PU.1, a master regulator of myeloid cell development, is significantly downregulated in AML. Our earlier findings that the autophagy-associated genes *MAP1S* and *WIPI1* are PU.1 transcriptional targets as well as our findings that knocking down *PU.1* significantly attenuates ATRA-mediated autophagic flux [42] prompted us to further investigate if additional ATG genes are regulated by this transcription factor. First, we determined ATG3, ATG4D, and ATG5 gene expressions in two independent NB4 *PU.1* knockdown cell lines upon ATRA treatment. Induction of all three genes was significantly reduced at the mRNA level when *PU.1* was knocked down (Figure 5(a)). To verify PU.1-dependent expression of these ATG genes, we used NB4 cells expressing an inducible PU.1-ER construct, which can be activated upon tamoxifen treatment leading to PU.1-ER translocation to nucleus and transcriptional activity. mRNA levels of all three genes were significantly induced upon PU.1-ER activation in NB4 cells (Figure 5(b)). We then analyzed a 3.5 kb genomic region up- and downstream of the transcriptional start site of all three ATG genes using MatInspector. We identified several putative PU.1 binding sites (Figure 5(c), left panel). PU.1 chromatin immunoprecipitation revealed binding of

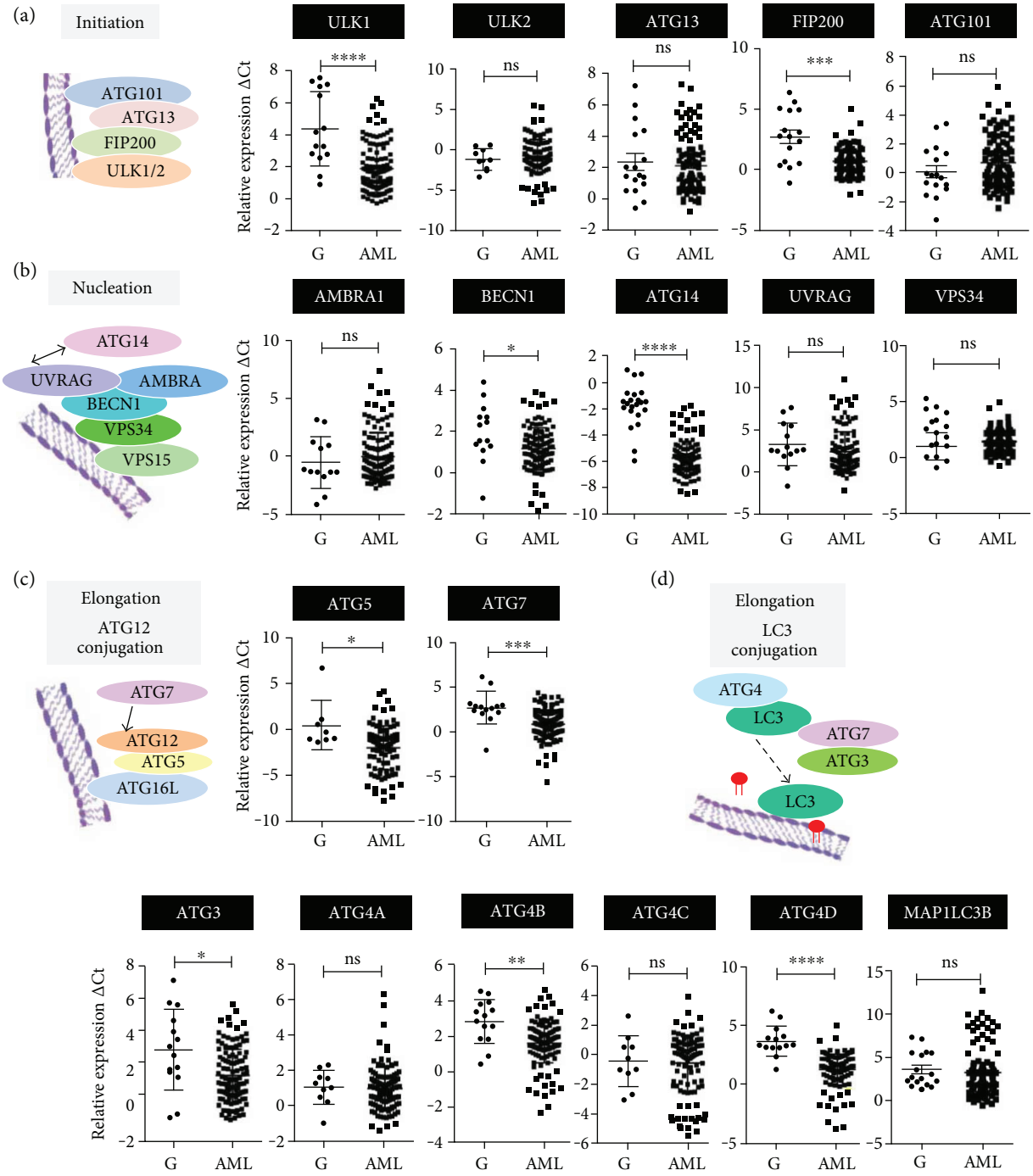


Figure 2: Autophagy- (ATG-) related gene expression is frequently inhibited in clinical AML samples. Primary AML blasts were isolated using a Ficoll gradient; total RNA was extracted; and ATG gene mRNA levels during autophagy initiation (a), nucleation (b), ATG12- (c), and LC3 (d) conjugation phases were quantified by qPCR. Measured cycle threshold (Ct) values represent log2 expression levels. Values were normalized to the expression levels of the housekeeping genes HMBS and ABL1. Mann–Whitney U test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

PU.1 to the promoter region of *ATG3*, *ATG4D*, and *ATG5* (Figure 5(c)). In summary, we identified three additional ATG genes that are regulated by PU.1 during ATRA-induced neutrophil differentiation of AML cells.

2.6. Posttranscriptional Regulation of ULK1 by miR-106a during AML Differentiation. Given the widespread aberrant microRNA (miRNA) expression in myeloid malignancies, we asked if altered miRNA expression in AML might contribute to ATG gene repression in this disease. Based on a miRNA profiling study that identified the miR-17 and miR-181 family members as the most downregulated miRNAs during neutrophil differentiation of NB4 APL cells [52], and our study identifying *ULK1* as a novel target of the

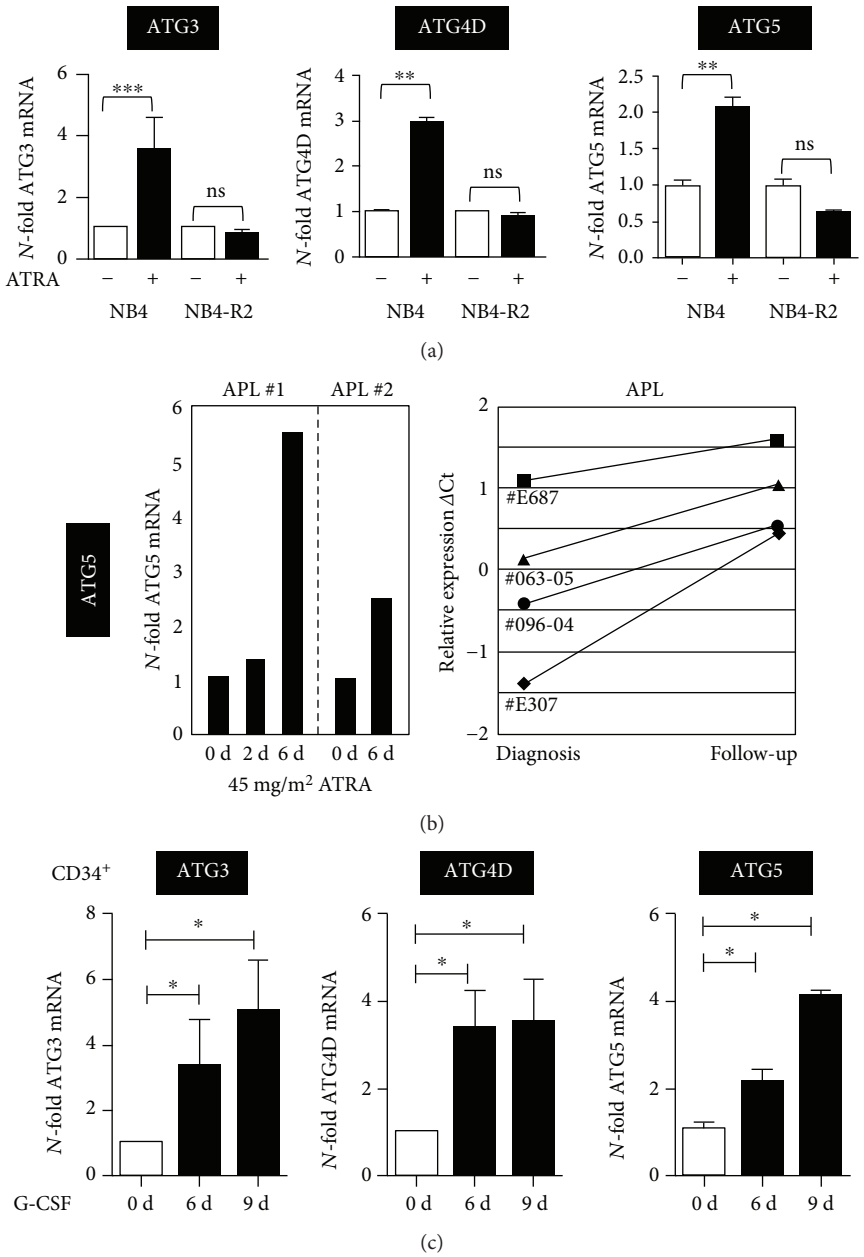
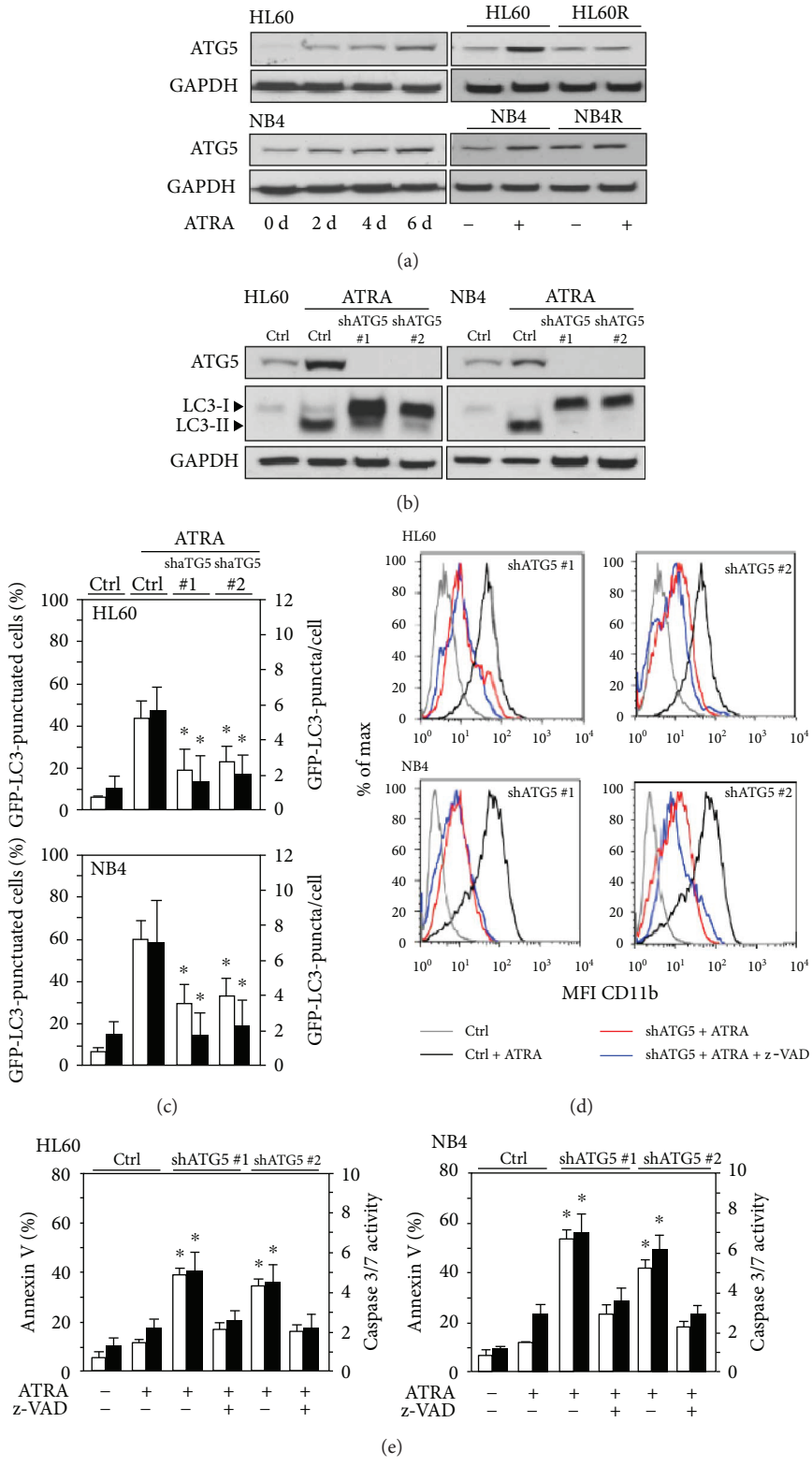


Figure 3: Induction of *ATG3*, *ATG4D*, and *ATG5* gene expressions upon neutrophil differentiation of normal and leukemic precursor cells. (a) *ATG3*, *ATG4D*, and *ATG5* qPCR analyses of NB4 and ATRA-resistant NB4-R2 cells upon treatment with 1 μM ATRA for 4 days. Results are given as *N*-fold changes compared with untreated SHC002 control cells and normalized to the housekeeping gene *HMBS*. (b) Induction of *ATG5* during ATRA therapy of APL patients. Two patients with newly diagnosed APL t(15;17) were treated with orally administered tretinoin (ATRA) at a dosage of 45 mg/m^2 daily. Total RNA was extracted from blast cells isolated using a Ficoll gradient, and expression levels of *ATG5* and were assessed by qPCR. Values were normalized to *HMBS* and day 0 as the experimental starting point (left panel). A similar induction of *ATG5* mRNA was seen in 4 APL patients at diagnosis and after finishing ATRA therapy (mean 1.3 months; right panel). Relative expression is given as differences in Ct values between *ATG5* mRNA and the levels of the housekeeping genes HMBS and ABL1 (ΔCt) representing log2 expression levels. (c) Normal CD34$^+$ progenitor cells were differentiated with G-CSF for indicated days; and *ATG3*, *ATG4D*, and *ATG5* mRNA expressions were determined by qPCR. Data analysis as in (a). Mann–Whitney *U* test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

miR-17 family member miR-106a in lung cancer therapy [53], we hypothesized that this miRNA also targets *ULK1* in AML. In a first step, we evaluated if knocking down *ULK1* similar to the other ATG genes analyzed would attenuate neutrophil differentiation. Indeed, using two independent shRNAs targeting ULK1, we found that inhibiting ULK1 resulted in significantly reduced *CEBPE* and CD11b levels (Figure 6(a)). Moreover, miR-106a expression prevented ULK1 induction upon ATRA-induced differentiation of NB4 paralleled by impaired neutrophil differentiation



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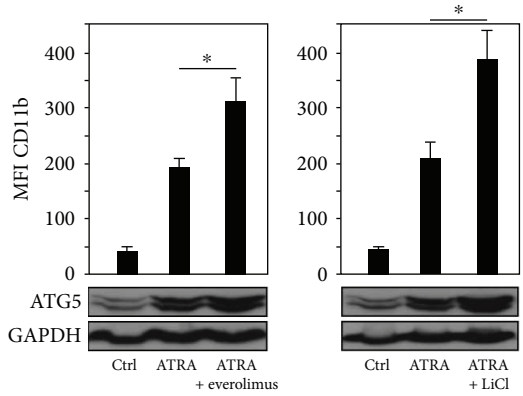

(f)

Figure 4: ATG5 induction is essential for neutrophil differentiation AML cells. (a) ATG5 protein expression in HL60, NB4, and the ATRA-resistant NB4-R2 and HL60-R upon ATRA-induced neutrophil differentiation. Cells were treated with 1 μM ATRA for up to 6 days. At time points indicated, proteins were extracted and ATG5 levels were analyzed by Western blotting. GAPDH was used as a loading control. (b) Inhibition of ATG5 precludes ATRA-induced autophagy. Cells stably expressing a scramble control shRNA (Ctrl) or shRNAs targeting *ATG5* (shATG5_1 and shATG5_2) were treated with ATRA for 4 days. Western blotting for ATG5 and LC3B is shown. GAPDH was used as a loading control. (c) Inhibition of ATG5 prevents ATRA-induced autophagy as measured by GFP-LC3 dot formation. NB4 and HL60 GFP-LC3 cells expressing scramble control shRNA (shCtrl) or shRNAs targeting *ATG5* (shATG5_1 and shATG5_2) were treated as in (b). The percentage of GFP-LC3 puncta-positive cells and average numbers of puncta were quantified by confocal microscopy. Counts are mean ± s.e.m.; $n = 100$; three independent experiments. (d) Inhibition of ATG5 impairs neutrophil differentiation of AML cells. FACS analysis of CD11b expression in cells treated as in (b) is shown. Blocking apoptosis by z-VAD-fmk did not alter reduced differentiation in ATG5 knockdown cells. Data are mean ± s.e.m.; $n = 1 \times 10^4$. (e) HL60 and NB4 *ATG5* knockdown AML cells displayed increased apoptosis upon ATRA treatment. Apoptosis was determined by annexin V staining and caspase 3/7 activity. z-VAD-fmk treatment efficiently attenuated apoptosis induction in *ATG5* knockdown cells during ATRA-induced differentiation. Cells treated as in (b). (f) Pharmacological activation of autophagy enhances neutrophil differentiation of AML cells. HL60 cells were treated with 1 μM ATRA alone or in combination with 0.5 μM everolimus (left panel) or 25 mM lithium chloride (right panel) for 4 days. The combination treatment significantly enhanced neutrophil differentiation as measured by CD11b induction. ATG5 induction was assessed by Western blotting. Data are mean ± s.e.m. of three independent experiments. Mann–Whitney *U* test, $^*p < 0.05$; n.s.: not significant.

as seen by significantly reduced induction of *CEBPE* mRNA compared to control transduced cells (Figures 6(b) and 6(c)). The expression of the known miR-106a target $p21^{CIP1}$, a gene induced during neutrophil differentiation, was determined as a positive control for the functionality of the miR-106a vector used (Figure 6(d)). Ectopic expression of miR-106a in NB4 cells is shown in Figure 6(e). Accordingly, using an anti-miR-106a construct, we found that blocking miR-106a resulted in increased protein expression of ULK1 during ATRA treatment whereas overexpression of miR-106a resulted in markedly reduced ULK1 expression (Figure 6(f)). Lastly, our preliminary data indicate that *ULK1* mRNA is negatively associated with miR-106a expression in a cohort of 16 primary AML patient samples (Figure 6(g)). Our studies clearly indicate that ULK1 is functional in neutrophil differentiation of AML cells and that it is targeted by the oncogenic miR-106a, providing a possible explanation for low ULK1 expression levels in AML.

3. Discussion

In this study, we identified a Beclin1-independent, low-intensity autophagy during neutrophil differentiation of AML cells. We determined the expression of key ATG genes involved in different autophagy phases in a large panel of primary AML patients. Overall, we found that the expression of a variety of ATG genes is significantly lower in primary AML as compared to normal granulocytes. The expression of these ATG genes is restored in AML cells upon induction of neutrophil differentiation paralleled by an activation of the autophagic flux. Moreover, we found that the Ets family transcription factor PU.1, a master regulator of myeloid differentiation, positively regulates expression of several ATGs. These findings add ATGs to the list of PU.1-regulated genes that are important for neutrophil differentiation. Thus, decreased ATG gene expression can partially be attributed to low expression of PU.1 in AML patients. We also showed that miR-106a targets the important autophagy gene ULK1 in AML. Together, we provide first explanations for low ATG expression in AML cells.

The role of autophagy during cell differentiation and mammalian development [18] has been long appreciated, and several studies show that disruption in autophagy function contributes to a cellular differentiation block. For example, autophagy is needed for elimination of mitochondria in red cell precursors, and deficiency in autophagy genes impairs erythrocyte maturation and causes anemia in mice [54]. During megakaryopoiesis and thrombopoiesis, autophagy is required for proper cell cycle and mitochondrial function, and a study using Atg7-deficient mice shows that autophagy deficiency causes impaired platelet production and function [43]. Accordingly, megakaryocytic differentiation of the chronic myelogenous leukemia

cell line K562 needs increased autophagy function [55]. During monocyte-macrophage differentiation, autophagy is induced upon release of Beclin1 from Bcl-2 and promotes not only cellular differentiation but also cell survival [45]. Another study using conditional *Atg5* knockout in myeloid progenitors described a mild expansion of precursor cells [56]. We and others showed that autophagy is crucial for therapy-induced neutrophil differentiation of AML cells indicating a similar dependence on autophagy for neutrophil development as in healthy individuals [38–40, 42]. Whether there are different functions for ATG5 or noncanonical autophagy in general during normal versus leukemic neutrophil differentiation is still under investigation.

The phosphoinositide-3-kinases (PI3K)/AKT and mTOR signaling pathways are currently targeted in clinical trials to treat AML by increasing cell death in combination with chemotherapy [57]. Since our results showed decreased ATRA-mediated differentiation when autophagy is inhibited, autophagy modulation in cancer therapy could have a potential negative effect on neutrophil differentiation. In general, class I PI3K inhibits autophagic initiation, whereas the class III enzymes stimulate autophagic activity. The net effect of broad-spectrum PI3K inhibitors targeting both classes of PI3K typically induces a block in autophagy. This block in autophagy might interfere with differentiation therapy. Nevertheless, specific inhibitors of class I PI3K had no impact on ATRA differentiation of APL cells and might be considered to enhance cell death in combination with chemotherapy [58]. Concerning our findings, we are confident that reduced ATRA-induced autophagy and neutrophil differentiation upon cotreatment with ATRA and 3-MA/wortmannin resulted in the inhibition of class III enzymes leading to a block of autophagy initiation. Our hypothesis is supported by earlier findings showing that knocking down *PIK3C3* resulted in a similar differentiation block as seen with 3-MA treatment [42]. Why does inhibition of *PIK3C3* but not of Beclin1 attenuate neutrophil differentiation despite that both proteins are part of the autophagy-initiation complex? Beclin1 might not be the limiting factor for the PIK3C3 nucleation complex in ATRA-induced autophagy. In favor of this explanation, we observed an increase in ATG5-ATG12-conjugated protein upon inhibition of Beclin1 (data not shown), suggesting that cells compensate the loss of Beclin1 by upregulation of other autophagy-related proteins. Similarly, small amounts of Beclin1 protein, which are released from Bcl-2 inhibition through ATRA-mediated downregulation of Bcl-2 [59], could be sufficient to activate and stabilize the PIK3C3 complex. Further, *PIK3C3* not only is involved in autophagosome nucleation but also acts at several steps along the signaling pathway associated with autophagy [60]. Thus, it seems possible that the *PIK3C3* knockdown phenotype during neutrophil differentiation represents the consequences of blocking autophagy at later stages.

Our findings in HL60 non-APL cells suggest that AML differentiation requires functional autophagy not only for the degradation of PML-RARA found in APL. Blocking autophagy interferes with differentiation, for example, with cell cycle arrest (data not shown), and results in cell death. Autophagy may protect malignant HSCs with a normal autophagy machinery or cancer cells under stress. However, autophagy is also important for maintenance of normal HSCs [61, 62]. Inhibition of autophagy by Bafilomycin A1 had no impact on differentiation, and VitD3 triggered autophagy resulting in Beclin1-dependent cell death. In line with previous finding that inhibition of autophagy results in apoptosis of cells that are engaged in differentiation [63], we now show a beneficial effect of cotreatment with ATRA and autophagy inducers. This differentiation-enhancing effect by combined activation of differentiation and autophagy might have clinical implications. A considerable number of APL patients present with major complications during differentiation therapy (e.g., ATRA syndrome or APL differentiation syndrome, 10–15%) [64]; thus reducing ATRA concentration in combination with autophagy activators might be beneficial for these patients. Generally, enhancing autophagy with FDA-approved drugs may represent a possible new strategy to treat APL patients and possibly sensitize additional AML subtypes to ATRA treatment. Promising autophagy-enhancing compounds to use in combination with ATRA include rapalogs (sirolimus, temsirolimus, and everolimus) and Ca^{2+} channel blockers (verapamil, loperamide, and pimozide) [65].

In conclusion, our studies show that increased ATG gene expression is associated with normal neutrophil differentiation, and that differentiation of AML cells involves a noncanonical autophagy pathway, which is Beclin1-independent. Increasing levels of ATG3, ATG4D, and ATG5 mRNA during neutrophil differentiation of healthy $CD34^+$ hematopoietic progenitor cells and their high expression in mature neutrophils point to a more general role for autophagy not only during leukemic but also during normal neutrophil differentiation. Key autophagy genes such as *ULK1*, *ATG3*, *ATG4D*, or *ATG5* are significantly downregulated in primary AML patient samples, and their expressions can be restored upon ATRA therapy in APL patients and AML cell lines. The low expression of these genes is partially due to inhibition of their positive regulator PU.1, or in the case of ULK1, by increased expression of its negative regulator miR-106a. Clearly, ATRA-induced myeloid autophagy is different from starvation- or chemotherapeutic-induced autophagy, and further investigations are needed to clarify its functions and to elucidate the signaling pathways involved. This is an important task since knowing the exact noncanonical autophagy pathway will serve to develop more specific and improved autophagy drugs. Finally, clinical applications planning to inhibit autophagy in order to decrease cell survival may need to consider detrimental effects on myeloid differentiation.

4. Materials and Methods

4.1. Primary Patient Samples, $CD34^+$ Cells, and Cell Lines. Primary AML patient cDNA samples were obtained from a cohort of AML patient samples from HOVON/SAKK (Dutch-Belgian Hematology-Oncology/Swiss Group for Clinical Cancer Research Cooperative group) protocols 04, 04A, 29, and 42 (available at http://www.hovon.nl) between

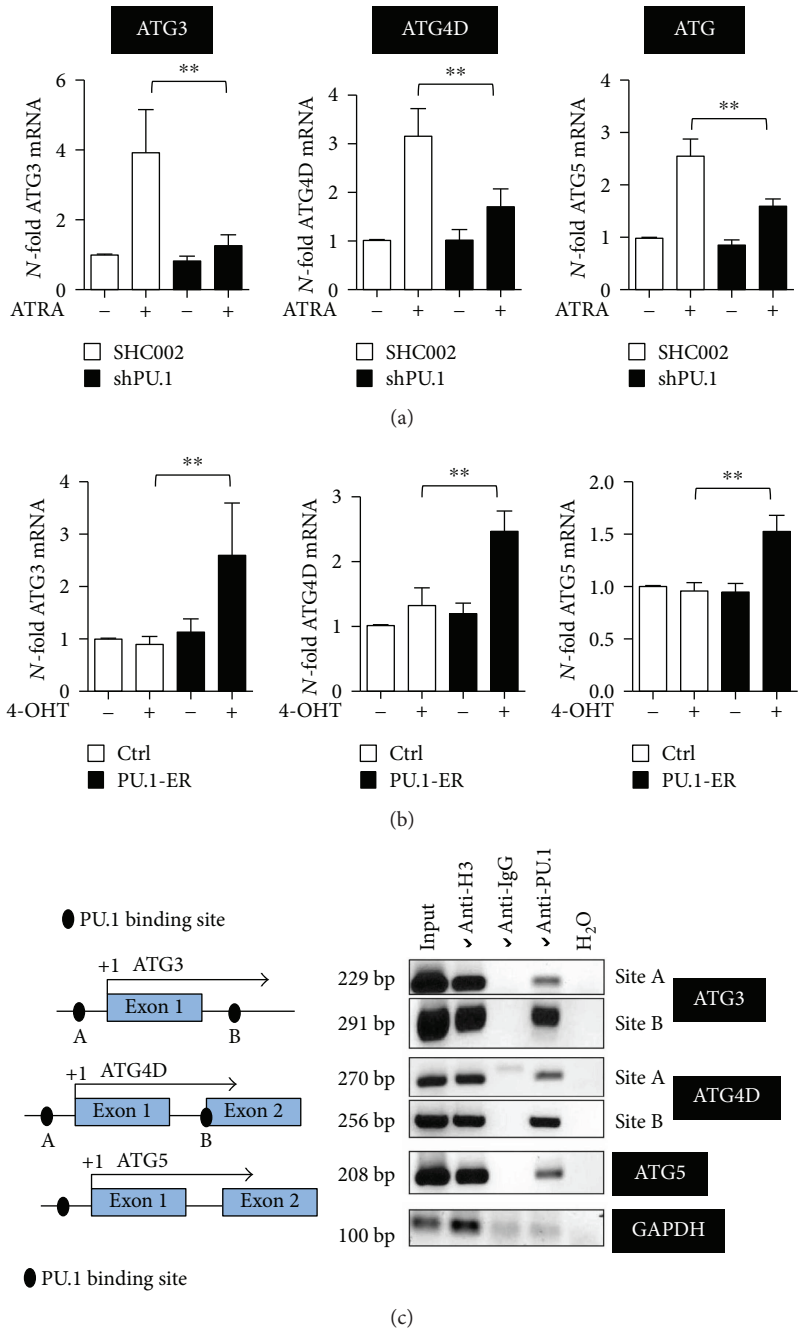

Figure 5: PU.1-dependent regulation of *ATG3*, *ATG4D*, and *ATG5* during ATRA-mediated differentiation of NB4 cells. (a) *ATG3*, *ATG4D*, and *ATG5* mRNA expression levels were quantified in NB4 shPU.1 cells treated with ATRA for 4 days. (b) NB4 cells, transduced with an inducible PU-1-ER expressing vector, were treated with 4-OHT to induce PU.1 translocation to the nucleus. *ATG3*, *ATG4D*, and *ATG5* mRNA expression levels were quantified as in (a). (c) Schematic representation of *ATG3*, *ATG4D*, and *ATG5* proximal promoter regions. Putative PU.1 binding sites in these promoter regions are indicated as black circles. *In vivo* binding of PU.1 to the indicated PU.1 binding sites was shown by ChIP in NB4 cells using antibodies against PU.1. Antibodies against acetyl-histone H3 and IgG are used as positive and negative controls, respectively. GAPDH amplification was shown as a negative control for the different pull-downs. Mann–Whitney *U* test, $^{**}p < 0.01$.

1987 and 2006. All patients provided written informed consent in accordance with the Declaration of Helsinki. Patient data represent log2 expression levels and were normalized to the expression levels of the 2 housekeeping genes *HMBS* and *ABL*. $CD34^+$-mobilized peripheral blood cells from healthy donors were expanded, cultured, and differentiated. The human APL cell lines NB4 and its ATRA-resistant clone NB4-R2, and the AML M2 cell line HL-60 and its ATRA-resistant clone HL60-R were maintained in RPMI-1640 (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich), in a humidified incubator containing 5% CO_2 at 37°C. The human embryonic kidney 293T cells were cultured in DMEM (Sigma-Aldrich) supplemented

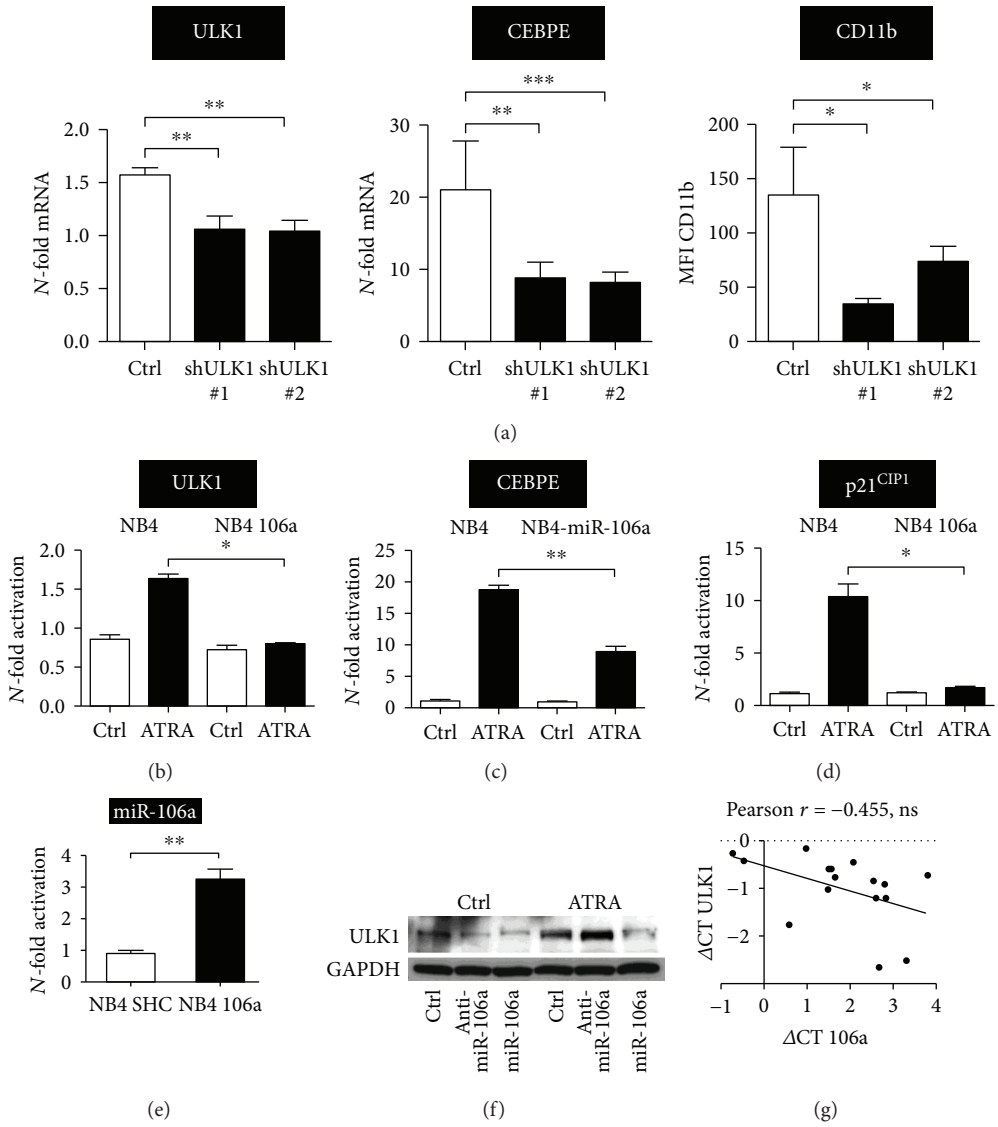

Figure 6: miR-106a targets *ULK1* and attenuates ATRA-induced AML differentiation. (a) Inhibition of ULK1 attenuates neutrophil differentiation of APL cells. NB4 cells stably expressing a scramble control shRNA (Ctrl) or shRNAs targeting *ULK1* (shULK1 #1 and shULK1 #2) were treated with ATRA for 4 days. qPCR for *ULK1* and *CEBPE* as well as FACS analysis of CD11b is shown. Data are mean ± s.e.m. of three independent experiments. (b) *ULK1*, *CEBPE*, and $p21^{CIP1}$ expressions were detected by qPCR in NB4 cells stably transduced with a scrambled control (NB4) or a lentiviral vector expressing miR-106a precursors (NB4 106a). Cells were treated with 1 μM ATRA for 4 days. Results were normalized to *HMBS* and are shown as *N*-fold change relative to the corresponding untreated control cell line. (e) qPCR analysis of miR-106a expression in NB4 control and miR-106a overexpressing NB4 cells. (f) Western blot analysis of ULK1 expression in control, anti-miR-106a, and miR-106a-expressing NB4 cells upon ATRA treatment for 4 days. GAPDH was used as a loading control. (g) Total RNA was isolated from 16 AML patients, and qPCR was performed to determine miR-106a and *ULK1* mRNA levels, respectively. SNORA38B and 5s rRNA were used as reference RNAs for the microRNA qPCR and *HMBS* and *ABL1* as reference mRNAs for the mRNA qPCR. ΔCt values of *ULK1* and miR-106a were calculated and plotted against each other. Linear regression and Pearson *r* were calculated using Prism software. Mann–Whitney *U* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with 5% FBS, 1% penicillin/streptomycin, and 1% HEPES (Sigma-Aldrich) and kept in a humidified atmosphere containing 7.5% CO_2 at 37°C.

For neutrophil differentiation, AML parental and knockdown cell lines were seeded at a density of 0.2×10^6/ml and treated with 1 μM ATRA (Sigma-Aldrich) for 2–6 days as indicated. Neutrophil differentiation was assessed by increased CCAAT/enhancer binding protein epsilon (CEBPE) mRNA expression and by CD11b FACS analysis. Arsenic trioxide (As_2O_3; Sigma) was dissolved in 1 M NaOH and used at 6–12 μM. 3-Methyladenine (3-MA; Sigma) was dissolved in H_2O and used at 5 mM. Chloroquine diphosphate salt (CQ; Sigma) was dissolved in H_2O and used at 25 μM. Bafilomycin A1 was dissolved in DMSO, used at 200 nM, and added 2 hours before analysis.

4.2. Lentiviral Vectors, Lentivirus Preparation, and Transduction of Cell Lines. pLKO.1 lentiviral vectors expressing small hairpin (sh) RNAs targeting PU.1, ATG5, or Beclin1 and a nontargeting shRNA control (SHC002) were purchased from Sigma-Aldrich. These vectors contain a puromycin antibiotic-resistant gene for selection of transduced mammalian cells. PU.1-ER construct was generated by subcloning PU.1-ER fragment into pLV-EF1a-MCS-IRES-Hyg vector containing hygromycin antibiotic-resistant gene using the In-Fusion HD cloning kit (Takara) according to the manufacturer's instruction. Lentivirus production and transduction were done as described [66]. Transduced AML cell line populations were selected with 1.5 μg/ml puromycin for 4 days or with 250 μg/ml hygromycin for 10 days. Knockdown efficiency was assessed by Western blot and/or qPCR analysis. An mCherry-EGFP-LC3-expressing lentiviral vector was kindly provided by Dr. Maria S. Soengas (CNIO, Molecular Pathology Program, Madrid, Spain).

4.3. RNA Extraction and Quantitative RT-PCR (qPCR). Total mRNA was isolated using miRCURY™ RNA isolation kits (Exiqon) according to the manufacturer's instruction. Total RNA was reverse transcribed, and ATG gene expression in AML patients was quantified using RT-PCR low-density arrays to quantify ATG gene expression as described previously [67]. For quantification of *ATG3*, *ATG4D*, *ATG5*, and *CEBPE* expression in cell line experiments, the TaqMan® Gene Expression Assays Hs00223937_m1, Hs00262792_m1, Hs00169468_m1, and Hs00357657_m1 were used, respectively. Specific primers and probes for *HMBS* and *PU.1* have been described [66]. miRNA expression was assessed using the miScript SYBR Green PCR kit and primer assay hsa-miR-106a (Qiagen). We used hsa-miR-SNORA-73A as a housekeeping gene for miRNA normalization. *N*-fold changes were calculated using the ΔΔCt method of relative quantification. Data represent the mean ± s.e.m. of at least triplicate experiments.

4.4. Western Blot Analysis. Whole cell extracts were washed in ice-cold PBS and lysed using urea lysis buffer consisting of 8 M urea and 0.5% Triton X-100 supplemented with protein inhibitor cocktail (Roche Diagnostics). The lysates were sonicated for 3 s and then centrifuged at 13,000 rpm for 15 minutes at 4°C. Bradford assay with BSA as a standard was used to determine the concentration of protein contents. 40 μg of total proteins was analyzed by electrophoresis on precast gel (Biorad). Blots were incubated with the primary antibody anti-LC3B (NB600-1384; Novus Biologicals) in TBS 0.05 Tween-20/5% milk or with anti-PU.1 in TBS 0.05 Tween-20/3% BSA overnight at 4°C and then incubated with secondary antirabbit DyLight 650 for 1 hour at room temperature. Additional antibodies used were anti-Beclin1 (Cell Signaling, #3738), anti-ATG5 (Sigma, A2856), and mouse monoclonal anti-GAPDH (Sigma). Blots were imaged using the ChemiDoc (Biorad) and Image Lab software.

4.5. Immunocytochemistry, Confocal Microscopy, and Image Analysis. We followed the methods of Wampfler et al. [68]. Briefly, NB4 cells were fixed in 2% paraformaldehyde in PBS and permeabilized in 0.1% Triton X in PBS or fixed and permeabilized in methanol (−20°C) after cytospin. Cells were then washed in PBS and incubated with primary LC3B antibody (Cat. No. 3686; Cell Signaling Technology) for 1 h at room temperature. Then, cells were washed twice in PBS-Tween and once with PBS, followed by incubation with the secondary antibody (FITC-conjugated antirabbit; Cat. No. 111-096-045; Jackson ImmunoResearch) for 1 hour at room temperature. Fluorescence-labeled cells were analyzed using a confocal laser scanning microscope, and quantification of LC3B dots was performed using ImageJ.

4.6. Autophagy and Apoptosis Assays. Autophagy was assessed by LC3 lipidation and GFP-LC3 redistribution. For GFP-LC3 dot formation, stable GFP-LC3-expressing AML cells were cytospun, fixed with 4% paraformaldehyde for 20 min at RT, washed with PBS, and covered with fluorescent mounting solution (Dako) prior to analysis by confocal microscopy (LSM510, Carl Zeiss). To quantify GFP-LC3 dots, at least 100 cells per slide in three independent experiments were assessed, that is, the percentage of GFP-LC3-positive cells with punctuate staining; and the number of discrete puncta per cell was counted.

Tandem mCherry-EGFP-LC3B-expressing cells were treated for 2 days with 1 μM ATRA. Data were acquired on a FACS LSR-II (BD) using BD FACSDiva software and analyzed with FlowJo software. A gate was used based on parental cells to estimate the percentage of high autophagic activity as previously described [49].

For annexin V staining, 0.5×10^6 cells were washed with cold PBS/5% BSA, resuspended in 70 μl binding buffer, and labeled with phycoerythrin- (PE-) labeled antibody against annexin V according to the manufacturer's protocol (BioVision). Caspase 3/7 activation was measured using Caspase-Glo™ 3/7 Assay according to the manufacturer's protocol (Promega Corporation, Madison, USA).

4.7. Statistical Analysis and Bioinformatics. Each value reported represents the mean ± SD of at least three independent experiments. *N*-fold changes were calculated using the −ΔΔCt method of relative quantification. Nonparametric Mann–Whitney *U* tests were applied to compare the difference between two groups using the program Prism software (GraphPad). p values < 0.05 were considered to be statistically significant. Promoter and gene sequences were retrieved from the online databases http://www.ncbi.nlm.nih.gov and http://www.ensembl.org/index.html. Putative PU.1 transcription factor binding sites were predicted by using MatInspector 8.0 software (http://www.genomatix.de).

4.8. Chromatin Immunoprecipitation Assay (ChIP). ChIP was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer's recommendation. Chromatin from 15×10^6 NB4 cells was fragmented to an average size of 500 bp using the provided enzymatic shearing cocktail. For immunoprecipitation, we used anti-PU.1 (sc-352; Santa Cruz Biotechnology) antibody. Immunoprecipitations with IgG (PP64B; Upstate)

or an anti-acetyl-histone H3 antibody (Stratagene) were used as negative and positive controls, respectively. Genomic regions containing putative PU.1 binding sites were amplified by PCR using the following primers: *ATG3* site A; forward 5′-3′: AGCATCAATCCACTCAGCATTC and reverse 5′-3′: CTGGATGGCAGTGGAAAAGAC; *ATG3* site B; forward 5′-3′: TCAGGGGTAAACTTGGAGCG and reverse 5′-3′: TTGGGATCGCAGTCACAACT; *ATG4D* site A; forward 5′-3′: CTGGAGCACTTCATTCATCCCT and reverse 5′-3′:TGAGACTGACTGCGCACC; *ATG4D* site B; forward 5′-3′: CGTTTTTGCCCCTCTCTGTA and reverse 5′-3′: CGGCTTTTAACCACCCAACC; and *ATG5*; forward 5′-3′: CAGCGTTGCCGGTTGTATTC and reverse 5′-3′: CTCCA GGCAACTACTCACCC. In addition, an unrelated sequence in the GAPDH gene was used as a negative control.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Jing Jin, Adrian Britschgi, Magali Humbert, and Anna M. Schläfli designed and performed the experimental research, analyzed the data, and drafted the article. Jasmin Batliner identified ULK1 as miR-106a target in AML differentiation. Marion Ernst, Elena A. Federzoni, and Deborah Shan-Krauer performed and analyzed ATG gene expression in primary AML patient samples and during granulocytic differentiation of $CD34^+$ progenitor cells. Bruce E. Torbett and Shida Yousefi instigated the experimental design, provided essential reagents as well as preliminary results, and revised the drafted article. Hans-Uwe Simon and Mario P. Tschan designed the project, analyzed data, wrote the paper, and gave final approval of the submitted manuscript. Jing Jin and Adrian Britschgi contributed equally to this work. Hans-Uwe Simon and Mario P. Tschan share corresponding authorship.

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Supplementary Materials

Supplementary 1. Supplementary Figure 1: attenuated neutrophil differentiation upon pharmacological inhibition of autophagy is not due to increased apoptosis. (a) 3-MA or CQ-mediated inhibition of autophagy impairs ATRA differentiation of HL60 cells. Cells were treated for 4 days with 1 μM ATRA alone or in combination with 5 mM 3-MA or with 25 μM CQ. CD11b was measured by flow cytometry as marker of neutrophil differentiation. In addition, apoptosis was blocked in the same setting using the pan-caspase inhibitor z-VAD-fmk. CD11b median fluorescence intensity (MFIs) data are mean ± s.e.m.; $n = 1 \times 104$. (b) 3-MA- and CQ-mediated inhibition of autophagy resulted in increased apoptosis in ATRA-treated HL60 cells. Apoptosis was determined by annexin V staining and caspase 3/7 activity. Cells treated as in (a). Data represent the mean ± s.e.m. of three independent experiments. Mann–Whitney *U* test, $^*p < 0.05$.

Supplementary 2. Supplementary Figure 2: ATG gene expression in AML patient subtypes. ATG gene mRNA expression levels during autophagy initiation (a), nucleation (b), ATG12 (c), and LC3 (d) conjugation phases were quantified by qPCR. Analysis as in Figure 2. Mann–Whitney *U* test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary 3. Supplementary Figure 3: ATRA-induced autophagy and neutrophil differentiation of AML cells is Beclin1-independent. (a) Minor Beclin1 protein induction during ATRA differentiation of NB4, HL60, and HT93 AML cells. Western blots of Beclin1 and GAPDH are shown. (b) Inhibition of Beclin1 did not abrogate ATRA-induced autophagy as measured by LC3B lipidation. Cells stably expressing a scramble control shRNA (shCtrl) or shRNAs targeting Beclin1 (shBeclin1) were treated with ATRA for 4 days. GAPDH was used as a loading control. (c) Inhibition of Beclin1 does not prevent ATRA-induced GFP-LC3 puncta formation. Left panels: HL60 and NB4 GFP-LC3 cells stably expressing a scramble control shRNA (shCtrl) or shRNAs targeting Beclin1 (shBeclin1) were treated with ATRA for 4 days. The percentage of GFP-LC3 puncta-positive cells and average numbers of puncta were quantified by confocal microscopy. Counts are mean ± s.e.m.; $n = 100$; three independent experiments; n.s.: not significant. Right panels: starvation- and arsenic trioxide- (As_2O_3) induced autophagy are inhibited by knocking down Beclin1. Cells were starved or treated with As_2O_3, and autophagic activity was assessed by confocal microscopy. Counts are mean ± s.e.m.; $n = 100$; three independent experiments. (d) Knocking down Beclin1 does not impair neutrophil differentiation of AML cells as determined by CD11b expression. Data are mean ± s.e.m.; $n = 1 \times 104$. Mann–Whitney *U* test, $^*p < 0.05$. n.s.: not significant.

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Review Article

The Role of Free Radicals in Autophagy Regulation: Implications for Ageing

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Reactive oxygen and nitrogen species (ROS and RNS, resp.) have been traditionally perceived solely as detrimental, leading to oxidative damage of biological macromolecules and organelles, cellular demise, and ageing. However, recent data suggest that ROS/RNS also plays an integral role in intracellular signalling and redox homeostasis (redoxtasis), which are necessary for the maintenance of cellular functions. There is a complex relationship between cellular ROS/RNS content and autophagy, which represents one of the major quality control systems in the cell. In this review, we focus on redox signalling and autophagy regulation with a special interest on ageing-associated changes. In the last section, we describe the role of autophagy and redox signalling in the context of Alzheimer's disease as an example of a prevalent age-related disorder.

1. Introduction

In parallel with the increase in mean human life span over the recent decades, interest has grown in better understanding the underlying mechanisms of ageing and their roles in pathological conditions with a view to extending health span. Ageing hallmarks include genomic instability, telomere attrition, epigenetic alterations, deregulated nutrient-sensing, cellular senescence, stem cell exhaustion, altered intercellular communication, mitochondrial dysfunction, and loss of proteostasis [1]. Notably, several of these hallmarks may be related to progressive alterations in oxidative metabolism and accumulation of oxidatively damaged proteins, lipids, and nucleic acids during ageing [2]. However, this relationship is more complex than originally believed, since it has become increasingly clear that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are not only detrimental to cells but can also have important roles as signalling molecules and participate in cellular functions such as cell-to-cell communication, proliferation, and survival in response to physiological cues and stress conditions [3].

Cellular functionality significantly depends on continuous maintenance and renewal of the whole proteome, that is, proteostasis (the loss of which defines one of the hallmarks of ageing). Two main cellular degradation systems are responsible for these functions: the ubiquitin-proteasomal system, which degrades individual proteins, and the autophagy-lysosomal system, which degrades whole organelles, protein aggregates, and long-lived proteins [4]. A prominent cause leading to the dysfunctionality of proteins is nonreversible oxidative modification. Proteolytic systems recognise and degrade such damaged proteins in order to prevent their accumulation and aggregation, thus preserving cellular viability. The general age-dependent decline in both proteasomal activity and autophagy, paralleled by accumulation of nondegraded dysfunctional material, has been reported in various mammalian models [5–9]. The

underlying reasons for this decline are still a matter of debate but they include changes in the composition/structure of the degradation systems themselves [10, 11], increased accumulation of the material designated for degradation, resulting in exhaustion of the degradation systems [12, 13], or a combination of both.

In this review, we summarise the most relevant findings that describe the age-related dysregulation of autophagy in the context of redox alterations. We also provide evidence of how the dysregulation of autophagy and redox homeostasis (redoxtasis) with age is closely related to the development of one of the most prominent age-related diseases of our time, Alzheimer's disease.

2. The Dual Role of Free Radicals

2.1. ROS/RNS as Signalling Molecules. ROS/RNS are produced during cellular metabolism, or in response to xenobiotics, cytokines, and bacterial invasion, and can be generated in mitochondria or other cellular structures (e.g., peroxisomes, endoplasmic reticulum, and phagosomes) by a variety of enzymatic reactions. Single-electron transfer to oxygen along the mitochondrial electron transport chain (mETC) leads to a small fraction of partially reduced oxygen in the form of superoxide anion, $O_2^{\bullet-}$ [14]. The prominent sites of superoxide formation are complex I (NADH dehydrogenase) and complex III (cytochrome bc1 complex). Other sources of $O_2^{\bullet-}$ production include the NADPH oxidase family which are multicomponent enzymes specialized in the production for $O_2^{\bullet-}$ in response to cellular stimuli involved in defense against pathogens or in cell proliferation [15]. Most other radical (hydroxyl radical $OH^{\bullet}$, $NO^{\bullet}$) and nonradical (H_2O_2) oxidative agents are derived from $O_2^{\bullet-}$. Furthermore, $O_2^{\bullet-}$ rapidly reacts with $NO^{\bullet}$ to form peroxynitrite ($ONOO^-$) in high yields [16]. In addition, H_2O_2 is produced as a by-product of fatty acid and amino acid oxidation in peroxisomes [17] and by the oxidation of protein dithiols by the thiol oxidase ERO1 in the endoplasmic reticulum [18]. Beyond this, nonenzymatic sources of radicals such as the Fenton reaction have also been documented. Metal ions in reduced oxidation states (Fe^{2+}, Cu^{+}) can induce the catalytic decomposition of hydrogen peroxide and the concomitant formation of hydroxide anions (OH^-) or extremely reactive hydroxyl radicals ($OH^{\bullet-}$). Their oxidized forms, Fe^{3+} and Cu^{2+}, can be reduced by various electron donors (including superoxides) to restore the redox-active state [19].

ROS/RNS are important second messengers in a number of signal transduction pathways critical for cell growth and proliferation [20]. ROS/RNS influence the activity of key cellular enzymes (tyrosine kinases, serine-threonine kinases, and protein phosphatases) by reversible oxidation of sensitive amino acids (cysteine and methionine) located in their catalytic domains [21–23]. Well-documented targets of ROS signalling are protein phosphatases that contain a redox-sensitive cysteine residue in their catalytic center, for instance, protein tyrosine phosphatase 1B (PTP1B), a negative regulator of the insulin-signalling cascade. The cysteine of the catalytic center can be oxidised to sulphenic acid, leading to transient PTB1B deactivation and further to a sulphenyl-amide intermediate, which may prevent irreversible oxidation and facilitate PTP1B reactivation [24]. ROS/RNS also regulate the transcription of many crucial genes via the modification of key regulators of NRF2, NFκB, HIF-1, and p53 transcription factors [25–28].

Another essential physiological function of ROS/RNS is the activation of the NLRP3 inflammasome upon infection by different pathogens [29–31]. Finally, ROS/RNS are critical mediators of cell death pathways, such as necrosis, apoptosis, and autophagy-programmed cell death [32]. The signalling function of ROS is facilitated by the existence of prominent redox sensors (mainly cysteines) within redox-regulated proteins known as "redox switches." As they are prone to transient oxidation, ROS can transiently change the activity or localisation of redox switch-containing proteins [4].

NO, a second messenger that can impact on several molecular targets, is prone to oxidation by superoxide. The highly reactive product, peroxynitrite ($ONOO^-$), can cause severe oxidative damage to biomolecules but can also potently modulate intracellular signalling by promoting the formation of 3-nitrosyl adducts with tyrosine moieties ("nitration"), as well as by less severe oxidation events. It is likely that nitration and oxidation of intracellular proteins by peroxynitrite are selective [33]. For instance, peroxynitrite can target receptor tyrosine kinase-signalling pathways [34]. Several mechanisms can mediate the peroxynitrite effects on tyrosine phosphorylation: (i) allosteric regulation of kinase activity by nitration or oxidation, (ii) modification of substrates, and (iii) peroxynitrite-induced modification (i.e., covalent dimerisation) of the receptors that subsequently attenuate kinase activity [35]. Regulation of receptor tyrosine kinases can occur due to modification of tyrosine phosphatase activity, as the active sites of many phosphatases contain cysteine thiolate, which normally serves as a transient acceptor of phosphate moieties but is inactive when oxidized by ROS/RNS. A typical example is PTP1B (previously referred to), which can be inactivated either by hydrogen peroxide or peroxynitrite. While H_2O_2 oxidation results in reversible cysteine oxidation to sulphenic acid, peroxynitrite catalyses irreversible sulphinic and sulphonic acid formation and terminal inactivation of the enzyme [35].

2.2. Redox Homeostasis (Redoxtasis). "Oxidative stress" was first formulated as a biological concept in 1985 [36] and has since enormously affected many areas of biological research. However, the meaning of the term has substantially changed over the years. The concept of oxidative stress was initially perceived as a simple imbalance between the formation of free radicals and their elimination by antioxidant defense systems. During subsequent decades, it became more and more apparent that free radicals are utilised as signals or regulators in many fundamental cellular processes. Thus, the concept of oxidative stress has now been updated to include the role of redox signalling and redefined as "a disturbance in the prooxidant-antioxidant balance in favour of the former leading to the disruption of redox signalling" [37].

Since proteins are the largest group of macromolecules, they are the most frequent targets of ROS/RNS. Thus, levels of protein carbonyls or nitrotyrosine are used as biomarkers

of oxidative stress [2]. Protein oxidation gradually results in the loss of activity, unfolding and exposure of hydrophobic patches, facilitating aggregation, and cross-linking and eventually rendering proteins resistant to proteolysis. Moreover, oxidative stress is closely related to the presence of advanced glycation end products (AGEs)—as a result of the chemical reaction between proteins and reducing carbohydrates—and to advanced lipid peroxidation end products (ALEs)—derived from the reaction between proteins and lipid peroxidation products. AGEs and ALEs represent a very heterogeneous class of molecules, which are formed by different pathways either exogenously (in food or tobacco smoke) or endogenously [38]. AGE- and ALE-modified proteins are characterised by the loss of structural and functional properties. For instance, glycated extracellular matrix proteins can inhibit the cell adhesion and migration of T-cells, accompanied by decreased actin polymerisation [39]. In addition to their direct, toxic effects, AGEs and ALEs can influence cell surface receptors. For example, the receptor for AGEs (RAGE) is expressed on the surface of various cell types [40] and mediates the induction of ERK and p38-MAPK signalling cascades [41, 42] as well as the activation of NADPH oxidase, enhancing ROS generation [43].

2.3. Cellular Mechanisms for Maintaining Redoxtasis. To protect themselves from excessive oxidative stress, organisms have developed a number of different response systems designed to sense and rapidly respond to changing levels of specific oxidants [44]. These mechanisms include (i) the endogenous antioxidant systems, (ii) transcriptional changes mediated by oxidative modification of specific transcription factors [45], (iii) activation of specific chaperones which protect against oxidative protein aggregation [46, 47], (iv) metabolism redirection (from energy production towards NADPH generation) by altering the activity of key enzymes involved in energy metabolism [48], and (v) activation of specific degradation systems (proteasomal degradation and/or autophagy) in order to eliminate damaged components.

2.3.1. Endogenous Antioxidant Systems. Endogenous antioxidant systems include low-molecular antioxidants such as vitamins, glutathione (reduced GSH and oxidized GSSG), lipophilic antioxidants, and uric acid, among others. Moreover, the electron donor groups, peroxiredoxins (PRXs), thioredoxins (TRXs), and glutaredoxins (GRXs) are considered guards of the intracellular redox state and key regulators of redox signalling.

Peroxiredoxins (PRXs) reduce hydrogen peroxides, organic hydrogen peroxides, and peroxynitrites [49]. They also translate information about the increased intracellular levels of oxidants into effector systems through the modification of signalling cascades. The catalytic active sites of PRXs and other thiol peroxidases contain cysteine, which is prone to oxidation by H_2O_2 and rapidly undergoes sulphenic acid formation [50]. Subsequently, this sulphenic acid reacts with thiol groups in target proteins, resulting in oxidation and regeneration, that is, the reduction of thiol peroxidase. The high redundancy of thiol peroxidases indicates their importance in cellular stress adaptations. Under conditions of high oxidative stress, sulphenic acid is further oxidised to sulphinic acid. Although "overoxidised" peroxiredoxins lose their antioxidant functions, they switch to molecular chaperones that expose their hydrophobic surfaces in order to bind protein-folding intermediates and prevent protein aggregation. The sulphinic acid in peroxiredoxins can only be reduced by mitochondrial sulphiredoxins [51, 52]. Thiol oxidation products can be reduced by thioredoxins (TRXs) or glutaredoxins (GRXs) [53]. TRXs prefer sulphenic acids, while GRXs can catalyse both S-glutathionylation and deglutathionylation, depending on the relative concentrations of GSH and GSSG. Under conditions where the GSH/GSSG ratio is decreased, that is, under the action of oxidising factors, GRXs can catalyse the S-glutathionylation reaction, while under weakening oxidative stress, GRXs can catalyse deglutathionylation [54, 55]. In contrast to the above-described redox switches, redox sensing in GRXs is dependent not only on reactive cysteines but also on Fe/S clusters stabilised by glutathione, which is derived from free-GSH pools [56–58]. GRXs may influence intracellular redox signalling by S-glutathionylation of effector proteins with different outcomes. S-glutathionylation catalysed by glutaredoxin has been shown to inhibit phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and PTP1B, among others. In contrast, proteins such as microsomal S-glutathione transferase, HRAS GTPase, and complex II of the mitochondrial respiratory chain are activated by S-glutathionylation [59].

2.3.2. Transcription-Dependent Control of Redoxtasis. An example of a redox-sensitive transcription factor is nuclear factor erythroid-derived-like 2 (NRF2). NRF2 activity is subject to a tight and multilevel control. The redox sensor KEAP1 enables NRF2 levels to adjust to oxidant fluctuations. Under basal conditions, NRF2 is sequestered by a KEAP1 homodimer, an E3-ligase that presents NRF2 to the CULLIN3/RBX1 protein complex, resulting in ubiquitination and proteasomal degradation. However, KEAP1 contains several key cysteines that can be oxidised, resulting in a conformational change that prevents the presentation of NRF2 to the proteasomal machinery and thus enabling newly synthesised NRF2 to accumulate and activate the expression of antioxidant response element- (ARE-) controlled genes. Several antioxidant and detoxifying enzymes as well as anti-inflammatory and proteostatic mediators are coded by NRF2-target genes [25, 60].

2.3.3. Chaperones. Oxidised proteins can lose their structure and become prone to aggregate. Thus, it is not surprising that specific chaperones are activated under oxidative stress conditions. The heat shock protein Hsp33 is normally inactive because of a highly conserved cysteine-containing zinc center. Oxidation leads to the formation of two intramolecular disulphide bonds accompanied by zinc release, which facilitates the formation of active, oxidised Hsp33 dimers. These dimers bind tightly to substrate proteins and prevent irreversible aggregation. Once redoxtasis is recovered, Hsp33 is reduced and the substrate protein is released [46, 47].

2.3.4. Degradation Systems. If redox imbalance exceeds the cellular antioxidant capacity, macromolecules and even organelles can suffer from oxidative damage. Fortunately, mammalian cells rely on a complex network of degradation systems, which guarantees the elimination of altered intracellular components such as oxidised proteins. The role of the ubiquitin-proteasome system (UPS) in the degradation of oxyproteins has been extensively addressed [4, 61–63]. In this review, we will focus on the autophagy process, which degrades not only soluble proteins but also aggregates and even organelles.

2.4. Autophagy and the Maintenance of Proteostasis. In addition to its importance in cellular recycling and energy supply during starvation, autophagy is now recognised as a critical housekeeping pathway in a broader range of conditions, including oxidative stress. The term "autophagy" encompasses all the processes by which cellular components (proteins, organelles, aggregates, and intracellular pathogens) are supplied to lysosomes for degradation. Different types of autophagy coexist in mammals, depending on the way in which cargoes are delivered to lysosomes. These mechanisms comprise macroautophagy, chaperone-mediated autophagy, and microautophagy.

2.4.1. Macroautophagy. Macroautophagy (often referred to as autophagy) is a process whereby portions of the cytoplasm are sequestered by the expansion and closure of compressed membranous cisterna (termed "phagophores"), to produce double- or multiple-membraned vesicles called "autophagosomes," which eventually fuse with lysosomes for degradation of the inner autophagosomal membrane and the sequestered content. Yeast studies have identified more than 30 autophagy-related proteins (ATGs) that are important for the autophagic process, and many of their orthologues have also been identified in mammals. The macroautophagic process in mammals is extremely complex, as different regulatory mechanisms can operate in distinct cell types and under different conditions in order to maintain proteostasis. Briefly, the ULK complex (formed by ULK1/ULK2-ATG13-FIP200-ATG10l) is activated by different signals, such as the energy sensor AMPK, enabling phagophore nucleation and assembly. The activated ULK complex targets and recruits a class III phosphatidylinositol-3 kinase complex (PI3K/VPS34-BECLIN1-VPS15-ATG14) to locally produce phosphatidylinositol-3-phosphate in the phagophore membrane, which serves to recruit other proteins to the nucleation site. The phagophore expansion step is associated with two ubiquitination-like reactions. First, ATG7 acts as an E1 ubiquitin-activating enzyme and ATG10 as an E2 ubiquitin-conjugating enzyme, enabling ATG12 conjugation to ATG5. Second, ATG12-ATG5 complexes interact noncovalently with ATG16L. This complex acts as an E3-ligase, facilitating the second ubiquitin-like reaction, where LC3 and GABARAP proteins are conjugated to phosphatidylethanolamine (PE) by ATG7 (E1-like) and ATG3 (E2-like) to form LC3-II and GABARAPs-II anchored to the phagophore membrane. Different autophagy cargo receptors, such as p62/SQSTM1 and NDP52, interact with ubiquitin-containing proteins as well as with LC3s and GABARAPs, enabling specific substrates to be engulfed by autophagosomes and delivered, through dynein-dependent movement along microtubules, to lysosomes, where fusion subsequently occurs (mediated by SNARE proteins). ATG12-ATG5 complexes dissociate from the autophagosomal membrane once autophagosome formation is complete, while ATG4 is necessary for the delipidation and recycling of LC3-II and GABARAPs-II, as well as for the initial proteolytic activation of newly expressed pro-LC3 and pro-GABARAP proteins. The resulting breakdown products inside lysosomes are released through permeases for recycling in the cytosol [64].

2.4.2. Chaperone-Mediated Autophagy (CMA). Chaperone-mediated autophagy (CMA) is a type of autophagy that facilitates the selective degradation of soluble proteins containing a specific KFERQ-like motif. The chaperone HSC70 recognises and binds to proteins bearing this pentapeptide [65]. HSC70 delivers substrate proteins to lysosomes, where they interact with the lysosomal receptor LAMP2A [66]. LAMP2A then multimerises and generates a translocon, which enables the susbstrate protein to enter the lysosome, assisted by lysosomal HSC70 (lysHSC70) and other sets of chaperones/cochaperones [67, 68].

2.4.3. Microautophagy. Microautophagy is the third type of autophagy in mammals and the least studied thus far. It involves the direct invagination of the lysosomal membrane, resulting in the engulfment of cytosolic cargoes that are then degraded by lysosomal proteases [69].

3. Redox Signalling and Autophagy Regulation

Many reports have demonstrated that redox signalling affects autophagic flux, generally resulting in its induction (reviewed in [61]). This may represent a cell survival mechanism, as autophagy enables the removal of damaged structures (protein and organelle homeostasis) and provides surplus energy substrates. For instance, upregulation of autophagy by rapamycin, lithium, carbamazepine, and valproic acid in SHSY5Y cells has been shown to protect against rotenone (a natural ROS-generating compound) toxicity in an ATG5-dependent manner [70, 71].

However, prolonged autophagy may result in the degradation of essential proteins and organelles and cell death (autophagic cell death or programmed cell death type II, PCD II) [72]. For example, increased SOD2-mediated H_2O_2 formation has been shown to facilitate autophagic, Atg5-dependent cell death in senescent keratinocytes through the accumulation of autophagic markers [73]. The outcome of autophagy induction is thus context-dependent, as it depends on the level, localisation, and type of ROS/RNS.

As the first line of defense against oxidative stress, posttranslational modification of key proteins of the autophagic-lysosomal pathway leads to an instant increase in autophagic flux. When oxidative stress becomes chronic, a long-term response can be generated via the activation of specific transcriptional networks (NRF2, NFκB, p53, and FOXO3). In addition to their effect on

autophagy machinery proteins (Figure 1), oxidants can also modify autophagy targets, thereby increasing or decreasing their susceptibility to degradation (Figure 2).

3.1. Redox Modification of Key Upstream Autophagy Regulators

3.1.1. 5′AMP-Activated Protein Kinase (AMPK). 5′AMP-activated protein kinase (AMPK) senses cellular stress and triggers the activation of several prosurvival pathways, including autophagy. AMPK may stimulate autophagy either indirectly via mTOR inhibition or directly via phosphorylation of ULK1 [74–76]. ROS and RNS can oxidise cysteine residues in both the α- and β-AMPK subunits, generating S-glutathionylated derivatives with increased kinase activity [77, 78]. Additionally, intracellular ROS can trigger Ca^{2+} release from the endoplasmic reticulum, while subsequent CaMKKβ activation also results in AMPK activation [79]. AMPK may be activated by hypoxia via ROS generated within the mitochondrial electron transport chain [80]. Moreover, starvation-induced ROS have been shown to induce AMPK-dependent autophagy, while cells overexpressing the antioxidant enzyme manganese-superoxide dismutase 2 (SOD2) fail to activate AMPK following starvation [81].

3.1.2. Ataxia Telangiectasia Mutated Protein Kinase (ATM). Ataxia telangiectasia mutated protein kinase (ATM) is a tumour suppressor protein crucial to the DNA damage-repair response. ATM exists in two different cellular pools: (i) nuclear ATM, involved in DNA repair and (ii) cytoplasmic ATM, which acts as a ROS sensor and an activator of the tuberous sclerosis complex 2 (TSC2) tumour suppressor by signalling to LKB1 and AMPK to relieve mTORC1 repression of autophagy [82]. The exact mechanisms employed by ATM in order to sense increased ROS concentration are yet unclear, but two possible mechanisms have been proposed. On the one hand, ATM contains many cysteine residues that are potential targets for direct oxidation by ROS. On the other, the signal may be mediated by oxidised lipid intermediates, since ATM colocalises with different membrane compartments in the cell.

3.1.3. Mitogen-Activated Protein Kinases (MAPK). Mitogen-activated protein kinases (MAPK) can be activated by ROS signalling, in a manner that can impact autophagy outcome. For instance, the kinase ASK1 binds to a reduced form of thioredoxin (TRX), which prevents dimerisation and activation. Oxidative stress promotes oxidation and dissociation of TRX and autophosphorylation of ASK1 and upregulation of its kinase activity [83]. ASK1 can phosphorylate and activate c-Jun N-terminal kinase 1 (JNK1). In turn, JNK1 can phosphorylate BCL-2 at multiple sites, thus disrupting its inhibitory interaction with BECLIN1 and favouring phagophore nucleation [84]. On the other hand, inhibition of JNK1 by NO reduces BCL-2 phosphorylation and increases the BCL-2-BECLIN1 interaction, thus inhibiting autophagy. It has also been observed that BCL-2 can be directly S-nitrosylated, which inhibits degradation and stabilises protein levels [85, 86].

ROS/RNS can also lead to sustained activation of the extracellular signal-regulated kinase (ERK) pathway, by either direct oxidation or nitration of the upstream activators RAF and MEK or inhibition of dual-specificity protein phosphatases or PP1/2A. ERK leads to phosphorylation and inactivation of TSC2, impairing its ability to inhibit mTOR signalling and thus suppresses autophagy [87].

3.1.4. KEAP1/NRF2. As previously mentioned, reactive cysteines in KEAP1 act as redox sensors, which, when oxidised, generate a conformational change in KEAP1 that renders it incapable of presenting NRF2 to the proteasomal machinery. As a consequence, NRF2 accumulates, translocates to the nucleus, and induces the expression of its target genes. The first direct link between NRF2 and autophagy was reported in connection with the autophagy receptor protein p62, which competes with NRF2 in binding to KEAP1 [88–90]. It has been suggested that the binding of p62 to KEAP1 leads to autophagic degradation of KEAP1, since silencing of p62 doubles the KEAP1 half-life [91, 92]. Phosphorylation of p62 increases its binding affinity to KEAP1, facilitating NRF2 accumulation and transcriptional activation of its target genes [88, 93]. It was also shown that TGF-β-activated kinase 1 (TAK1) can phosphorylate p62, enhancing KEAP1 degradation and NRF2 upregulation. TAK1 deficiency upregulates ROS in the absence of any exogenous oxidant in parallel with a reduction in NRF2 protein levels suggesting that TAK1/p62/NRF2 axis is a way to regulate cellular redoxtasis under steady-state conditions [94].

NRF2, in turn, regulates the expression of relevant genes for macroautophagy, including ULK1, p62, NDP52, ATG4D, ATG7, GABARAPL1, ATG2B, and ATG5 [93, 95–97]. Therefore, it seems that NRF2 activation increases macroautophagy, which in turn results in KEAP1 degradation and favours further NRF2 stabilization in a positive feedback loop. This mechanism of NRF2 induction might be a relevant response to prolonged cellular stress.

3.1.5. IKK/NFκB. Nuclear factor kappa-light-chain-enhancer of activated B-cell (NFκB) signalling and autophagy is reciprocally involved in the control of cellular survival under conditions of stress. In an unstimulated state, NFκB renders in the cytosol as an inactive complex with IκB. Various conditions of stress, including oxidative stress, activate upstream IκB kinases (IKK) that phosphorylate IκB, leading to its ubiquitination and proteasomal degradation. Autophagy induction by nutrient starvation or mTOR inhibition by rapamycin correlates with IKK activation and IκB degradation but not necessarily with the activation of NFκB [98]. Constitutive activation of the IKK complex involved hyperphosphorylation-dependent activation of AMPK, hypophosphorylation of the mTOR substrate $p70^{S6K}$, depletion of p53 protein, and release of BECLIN1 from the inhibitory complex with BCL-2. Autophagy induction by constitutively active IKK could be prevented by knockdown of the α-subunit of AMPK suggesting that IKK-stimulated autophagy is controlled by the canonical AMPK/mTOR pathway [98]. These data indicate that the IKK complex may induce autophagy,

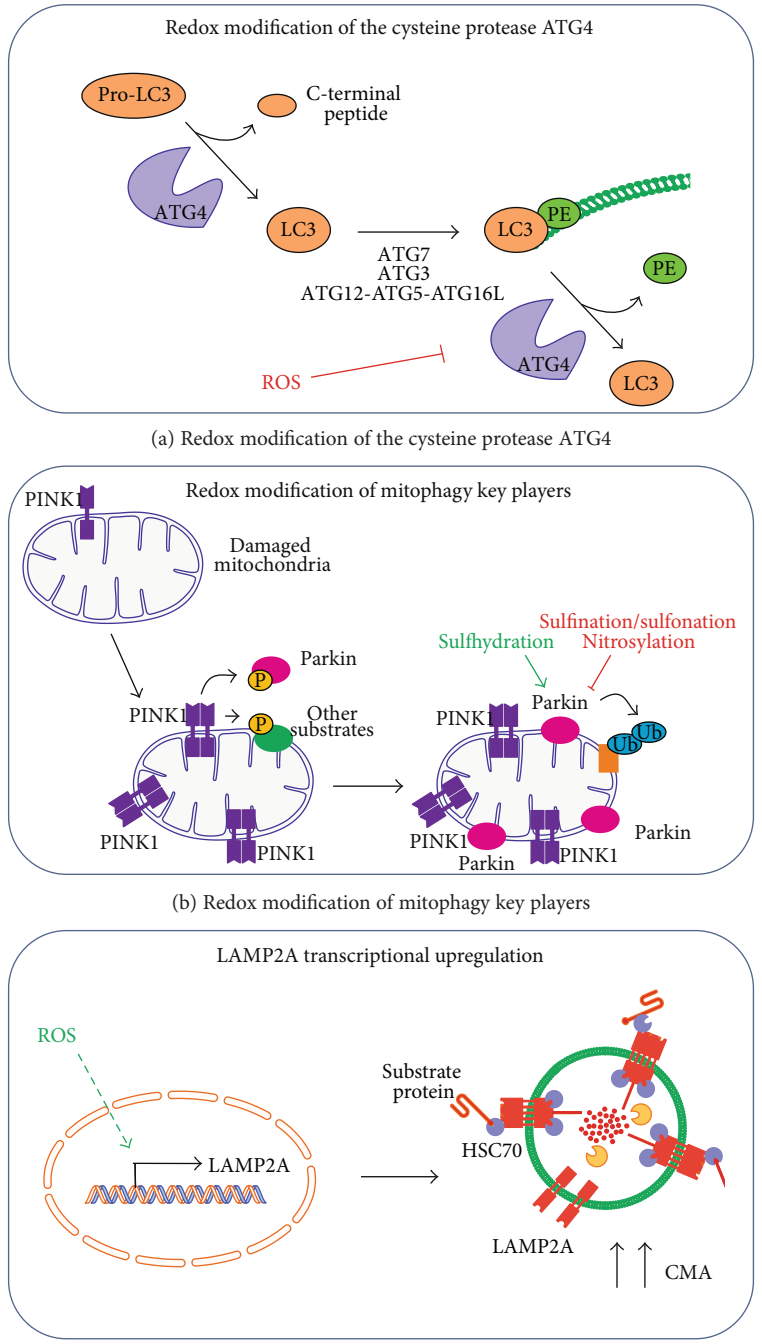

Figure 1: Redox modification of autophagy core components. (a) Cysteine protease ATG4 is sensitive to redox modification. ATG4 cleaves the C-terminal peptide in LC3 (or GABARAPs), making it a suitable substrate for conjugation to phosphatidylethanolamine (PE), which is mediated by ATG7, ATG3, and the ATG12-ATG5-ATG16L complex. LC3 conjugated to PE (LC3-II) is inserted into the autophagosomal membrane and enables it to elongate. ATG4 also acts as a delipidating enzyme, releasing LC3 from PE. ROS are essential for regulating ATG4 activity, as redox modification of cysteine residues transiently inhibits delipidation activity in order to promote autophagosome formation. (b) Mitophagy core components are targets of redox modification. Briefly, damaged mitochondria result in the stabilisation, dimerisation, and activation of kinase PINK1 in the organelle. PINK1 phosphorylates Parkin and other substrates, which further recruit Parkin to the mitochondrial membrane. Parkin acts as an E3-ubiquitin ligase, ubiquitinating several substrates that are recognised by autophagy receptors in order to direct mitochondria toward lysosomal degradation. Physiological sulfhydration enables, whereas pathological nitrosylation or sulphination/sulfonation inhibits, Parkin catalytic activity. (c) Mild oxidative stress upregulates chaperone-mediated autophagy (CMA) by transcriptional induction of lysosomal receptor LAMP2A.

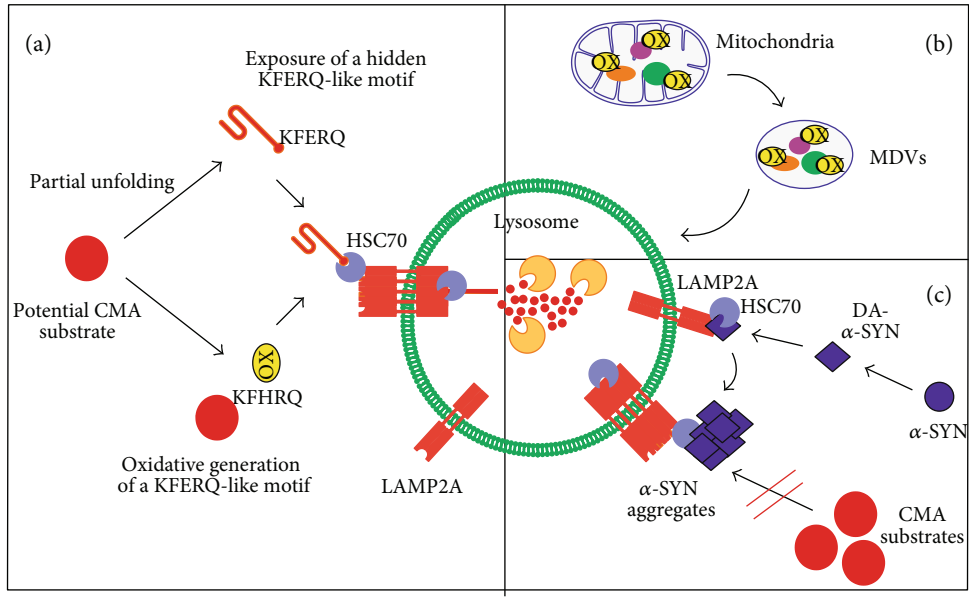

Figure 2: Redox modification of autophagy targets. (a) Oxidative-modified targets are better substrates for CMA degradation. Possible explanations for the increased degradation of oxidised substrates by CMA include (i) partial unfolding of substrates facilitating lysosomal translocation; (ii) partial unfolding of substrates exposing hidden KFERQ-like motifs; (iii) generation of a new KFERQ-like motif due to specific oxidation of amino acid residues. (b) The enrichment of oxidised substrates in mitochondrial-derived vesicles (MDVs) points to a mitochondrial quality control mechanism under oxidative stress conditions. (c) Specific redox modification of targets involved in disease. The interaction of oxidised dopamine with α-synuclein (α-SYN) generates dopamine-modified α-SYN (DA-α-SYN), which is poorly degraded by CMA; it instead forms oligomers and aggregates, further blocking the degradation of other CMA substrates.

but experiments with mouse embryonic fibroblasts knocked out for IKK subunits revealed that IKK is not indispensable [99]. More likely, IKK is required for the optimization of autophagy induced by physiological and pharmacological stimuli. During onset of stress conditions, IKK stimulates autophagy via NFκB-independent increased expression of ATG5, BECN1, and LC3 [100], but IKK itself can be inactivated by S-nitrosylation [85]. Conversely, autophagy may contribute to the regulation of the IKK pathway as all three IKK subunits (α, β, and γ) as well as their upstream activator NFκB-inducing kinase are degraded by the autophagic pathway [101].

Released NFκB can translocate to the nucleus and function as an efficient transcription factor [102]. The role of NFκB in autophagy regulation is ambiguous. On the one hand, it can promote autophagy by transactivating the proautophagy protein BECLIN1 [103]. On the other hand, TNFα was shown to repress autophagy via NFκB-dependent activation of the autophagy inhibitor mTOR in Ewing sarcoma cells. In cells lacking NFκB, TNFα treatment upregulated the expression of BECLIN 1 and subsequently induced an accumulation of autophagic vacuoles. Both of these responses were dependent on ROS production [104]. Interestingly, Atg5- and Atg7-deficient mouse embryo fibroblasts are unable to activate the NFκB pathway in response to TNFα, which points to a role of autophagy in NFκB activation [105].

NRF2- and NFκB-signalling pathways must be well coordinated in order to keep the fragile balance between the antioxidative and proinflammatory processes that occur in the cell. One of the underlying mechanisms of crosstalk depends on autophagy. Free KEAP1 can prevent IKK from binding to heat shock protein 90 (Hsp90) [106] thereby inducing autophagic degradation of IKK and attenuating NFκB signalling [107].

3.1.6. Sirtuin 1. Sirtuin 1 (SIRT1), a class III histone deacetylase, is a key component of the cellular prosurvival pathway during the response to stress conditions [108]. The excessive presence of ROS induces SIRT1 activation and translocation to the nucleus via two independent effector pathways, namely, JNK1 and AMPK [108]. Nevertheless, SIRT1 itself can be a target of oxidative modification specific of cysteine residues, which enhance its degradation by the proteasome [109]. Activated SIRT1 is a potent inducer of autophagy and exerts its effect either directly via interaction with components of the autophagy cascade or indirectly via FoxO signalling. SIRT1 can participate in a molecular complex with several essential components of the autophagy machinery, including ATG5, ATG7, and LC3. It can also deacetylate them, thus promoting autophagosome formation [110]. FoxO proteins belong to a family of transcription factors that are activated during conditions of cellular stress. SIRT1 deacetylates FoxO1, which subsequently stimulates expression of RAB7, a protein essential for autophagosome fusion with lysosomes [111]. FoxO3, another member of the FoxO family, can also be deacetylated by SIRT1 in response to oxidative stress, stimulating the expression of the autophagy proteins LC3 and BNIP3 in skeletal muscle [112].

3.2. Redox Modification of Autophagy Core Proteins

3.2.1. Autophagy-Related Protein 4 (ATG4). ATG4 is an important member of the autophagy cascade, and it is essential for autophagosome formation. It has the dual role of, first, cleaving LC3 and GABARAPs at the C-terminus so that they can be conjugated to phosphatidylethanolamine (PE) and, second, cleaving (deconjugating) LC3 and GABARAPs from the already-formed autophagosomal membrane. ATG4 contains reactive cysteines prone to oxidation by ROS (specifically by H_2O_2, generated upon starvation), which reversibly inhibit ATG4 activity [113]. It was proposed that starvation induces a local production of H_2O_2 in the vicinity of the autophagosome formation site [113]. This would locally inactivate ATG4, so that it cannot deconjugate PE-conjugated LC3 and GABARAPs on the phagophore, thus allowing autophagosome formation. As autophagosomes are trafficked towards lysosomes, they presumably arrive to environments with lower H_2O_2 concentrations, allowing ATG4 reactivation, and thus the deconjugation and recycling of LC3 and GABARAPs (Figure 1(a)).

3.2.2. Mitophagy Players. Mitophagy is a specific type of autophagy in which mitochondria are targeted for lysosomal degradation. The E3-ubiquitin ligase Parkin translocates to damaged mitochondria and is one of the key regulators of mitophagy induction [114]. Parkin contains two highly conserved cysteines. Their mutation has been linked to Parkinson's disease and results in the loss of Parkin activity and impaired mitophagy [115], which points to the importance of these redox-sensitive residues. A study by Meng et al. showed that sulphination/sulfonation of key cysteine residues in Parkin, as well as in protein regions affected by familial mutations, led to decreased activity of the enzyme and contributed to protein aggregation [116]. Moreover, Vandiver et al. reported that Parkin is physiologically sulfhydrated and that, whereas nitrosylation inactivates it, sulfhydration stimulates its catalytic activity [117] (Figure 1(b)). Another protein implicated both in the antioxidant response and in mitochondrial removal is DJ-1. Similar to what occurs with Parkin, DJ-1 is susceptible to redox signalling. Thus, oxidation of a specific cysteine in this protein is necessary for mitochondrial targeting and protection against oxidation-induced cell death [118].

3.2.3. LAMP2A. Although there have been no reports of a direct oxidative modification of the lysosomal receptor for CMA (LAMP2A), mild oxidative stress leads to increased LAMP2A levels, together with augmented lys-HSC70 and the cochaperones HIP and HSP90. In contrast to other CMA-activating stimuli, such as nutrient deprivation, increased LAMP2A levels under oxidative stress are achieved transcriptionally [119] (Figure 1(c)).

3.3. Redox Modification of Autophagy Targets

3.3.1. CMA Targets. CMA is required for preserving cell viability in response to oxidative stress. Thus, exposure of CMA-incompetent cells to oxidant and prooxidant factors (H_2O_2, paraquat, and cadmium) results in more severely compromised cell viability than in cells with preserved CMA function [120]. In fact, increased levels of oxidised proteins can be found in the lysosomal lumen under mild oxidative stress conditions, presumably due to the higher binding and uptake of substrates [119]. Incubation of well-known CMA substrates and a pool of cytosolic proteins with increasing amounts of prooxidants accelerate degradation by CMA. The mechanisms by which ROS facilitates degradation have not been fully elucidated. One possible explanation is that protein oxidation causes partial unfolding, not only exposing hidden recognition motifs to HSC70 but also facilitating translocation to the lysosomal lumen (Figure 2(a)). Another possibility is that oxidation of certain residues creates a previously nonexisting KFERQ-like motif. For instance, positive histidine when oxidized will resemble a negative aspartic acid residue [121].

3.3.2. Mitochondrial-Derived Vesicles (MDVs). A new mechanism for maintaining mitochondrial quality control, different from mitophagy, has recently been described [122]. Mitochondrial-derived vesicles (MDVs) are generated by a budding process from mitochondria in order to selectively transport mitochondrial proteins to either the peroxisomes or the lysosomes for degradation. MDVs are stimulated under different stress conditions and contain specific cargoes depending on the nature of the insult. Interestingly, Soubannier et al. showed enrichment in oxidised cargoes within these vesicles [123]. This process may represent a quicker mitochondrial quality control mechanism than mitophagy, as it preserves mitochondrial function by selectively degrading damaged mitochondrial proteins (Figure 2(b)).

3.3.3. Specific Proteins Involved in Disease. Specific individual proteins have been extensively analysed for oxidative modifications because of their involvement in disease. One example is α-SYN due to its aberrant accumulation in Parkinson's disease. Oxidation and nitration of α-SYN stabilises protein polymers by forming stable cross-linked α-SYN aggregates. Using HEK293 cells stably transfected with wild-type and mutant α-SYN, Paxinou et al. demonstrated that intracellular generation of nitrating agents results in the formation of α-SYN aggregates and prevents them from being degraded in lysosomes [124]. Dopaminergic neurons are thought to be particularly vulnerable to nitrosative/oxidative damage. Interestingly, a modified form of α-SYN, resulting from a noncovalent interaction with oxidised dopamine, has been suggested to be responsible for neuron toxicity [40]. While α-SYN is, at least in part, degraded by CMA [125], mutant and dopamine-modified forms of α-SYN are no longer properly degraded by this pathway. Instead, these forms of α-SYN tend to aggregate and prevent degradation of other substrates, further impairing proteostasis and increasing susceptibility to oxidative stress [125, 126] (Figure 2(c)). Other examples will be extensively analysed in the context of Alzheimer's disease (AD) in Section 5.

4. Changes in ROS Signalling and Autophagy with Ageing

Ageing is associated with the accumulation of oxidatively modified proteins. The final burden of dysfunctional proteins depends on multitude of factors that govern (a) the rates of formation of various kinds of ROS, (b) the levels of antioxidant defenses that guard against ROS-mediated protein damage, (c) the sensitivity of proteins to oxidative attack, and (d) the capacity of the cell to repair or eliminate damaged proteins [2].

4.1. Ageing and the Antioxidant Defense System. Although some studies support the premise that antioxidant enzyme function does not generally decrease with age [127], reduced capacity of specific antioxidant systems has been shown to develop with age [128]. One supporting example is the fact that levels of methionine sulphoxide increase with age in humans (i.e., in cataractous lenses, trabecular meshwork, skin collagen, or senescent erythrocytes), probably due to decreased methionine sulphoxide reductase (MSR) activities [129]. Interestingly, no increase in methionine sulphoxide was found in aged mouse tissues [129]. Studies performed in *D. melanogaster* or mice demonstrated that loss-of-function mutations in MSR correlates with reduced maximal life span, while MSR-overexpression results in extended life span [130–132]. This is also the case for transcription factor NRF2, the master regulator of the antioxidant cell response (reviewed by Bruns et al.) [133]. Reduced binding of NRF2 to its antioxidant response element (ARE) has been observed in aged rodents in parallel with reduced glutathione levels [134]. Studies in *D. melanogaster* have shown reduced NRF2/CncC responsiveness to stress. Overexpression of the NRF2/CncC partner Maf restored NRF2/CncC signalling competence and antagonised age-associated functional decline [135]. These and other studies support the hypothesis that the inability of the organism to adapt to internal and external conditions contributes to age-related loss of homeostasis [127, 135].

4.2. Macroautophagy Decline in Ageing. Plenty of evidence shows that a decline in the capacity of proteolytic systems occurs with age. In fact, the overall reduced rates of protein degradation with age were first observed almost three decades ago [136, 137].

The crucial role of autophagy in the ageing phenotype is reflected by studies in which loss-of-function mutations or deficient expression of several autophagy-related genes results in decreased life span in different organisms [8]. Matecic et al. performed an unbiased screen for ageing factors in the yeast *S. cerevisiae*, which led them to identify many short-lived mutants with autophagic defects [138]. Conversely, several reports demonstrate that the activation of macroautophagy (genetically, pharmacologically, or by calorie restriction) extends the life span of various organisms [139]. For example, brain-specific overexpression of Atg8 (the orthologue of LC3/GABARAPs) and treatment with spermidine has been shown to induce autophagy and extend life span in *D. melanogaster* flies [140, 141]. In mice, treatment with the autophagy activator rapamycin slows age-related alterations and prolongs longevity [142, 143]. Overexpression of ATG5 in mice resulted in extended life span along with antiageing phenotypes, including leanness, increased insulin sensitivity, and improved motor function. Interestingly, cultured fibroblasts from these mice were more resistant to oxidative damage in an autophagy-dependent manner [144]. In fact, it has been suggested that the long-lived naked mole rat copes with chronic oxidative stress by enhancing its proteostatic network [145].

Various studies have revealed a decrease with age in both the formation and subsequent elimination of autophagosomes in different tissues of aged animals [146, 147]. The levels of core autophagy proteins in various tissues from distinct aged organisms have been analysed. The expression of many components of the autophagy pathway is reported to be reduced with age in *Drosophila* muscles (i.e., Atg1, Atg5, Atg6, Atg7, and Atg8) [148]. LC3 and ATG7 levels have been shown to be downregulated in the muscles of aged mice and humans [149]. Another study reported downregulation of several autophagy-related genes (e.g., Atg5, Atg7, and BECN1) in the human aged brain [150]. Ott et al. reported reduced levels of ATG5-ATG12, LC3-II/LC3-I ratios, BECLIN1, and p62 in aged murine brain tissue and senescent human fibroblasts [151]. However, the precise mechanisms that lead to reduced expression of ATGs remain unclear. Dysregulation of signalling pathways that regulate autophagy may also contribute to the age-related decline in autophagy. For instance, the stimulatory effect of glucagon on macroautophagy is blunted with age, while the inhibitory effect of insulin remains intact [152].

4.2.1. Lipofuscin Accumulation. Defective autophagy may favour the accumulation of lipofuscin with age. Oxidised proteins may not undergo adequate proteolytic digestion but instead cross-link with one another or form extensive hydrophobic bonds [153]. These cross-linked proteins can react with other cellular components and generate an autofluorescent material called "lipofuscin," a nondegradable polymeric substance consisting of proteins (30–70%), lipids (20–50%), and sugar residues (7%) [154]. 99% of lipofuscin colocalises with lysosomes, whereas only 1% is found in the cytosol [4]. Although macroautophagy is responsible for the uptake of lipofuscin into lysosomes, experiments using an ATG5-knockout model showed that inhibition of macroautophagy does not prevent lipofuscin formation but rather leads to accumulation of cytosolic lipofuscin with enhanced cytotoxicity [155]. Lysosomes are the degradation site for iron-containing metalloproteins, such as cytochromes and ferritin, resulting in the release of redox-active low-molecular-mass iron. In ferrous form, ferritin reacts with hydrogen peroxide (which easily diffuses throughout the cell), forming the extremely reactive hydroxyl radical via the Fenton reaction. Hydroxyl radicals are highly unstable and react with fatty acids to form organic peroxides and aldehydes, which can react with one or two free amino groups within proteins, forming Schiff bases. The formation of aldehyde bridges, an important mechanism of protein-protein cross-linking, is involved in lipofuscinogenesis [156].

While mitotic cells are able to "dilute" lipofuscin via ongoing cell division, this pigment particularly accumulates in postmitotic tissues with age. In fact, lipofuscin can fill up to 40% of the cytosolic volume in aged animals. Lipofuscin-loaded lysosomes are no longer considered "residual bodies," as they have been shown to receive new lysosomal enzymes in an attempt to degrade lipofuscin. Nonetheless, the accumulation of lipofuscin in lysosomes also impairs efficient lysosomal degradation of other substrates. Indeed, lipofuscin-loaded human fibroblasts exhibit reduced autophagy under conditions of starvation [157]. Moreover, lipofuscin inhibits proteasomal activity [158] and is considered a source of oxidative stress because of the incorporation of transition metals. Overall, lipofuscin impairs degradation of other proteins and increases the potential for further oxidative damage [159].

4.2.2. Formation of Advanced Glycation Products. Several studies have confirmed the accumulation of AGEs and ALEs with age in different tissues, including rodent and human skin [136], eye lens [160], renal arteries [161], and intervertebral discs [161]. Many studies indicate that the amount of AGEs in certain tissues correlates with the half-lives of their proteins. For example, higher levels of AGEs have been found in cartilage collagen (half-life of 117 years) compared to skin collagen (half-life of 15 years) [162]. It would seem that intracellular proteins are protected from transformation to AGEs because of their fast turnover. However, the proteolytic capacity of the cell decreases with age, making proteins more susceptible to glycation. Furthermore, lysosomal proteases may be inhibited by glycating agents and AGE-modified proteins, which will limit degradation and allow accumulation of AGEs [163, 164].

4.2.3. Transcriptional Regulation. Transcriptional regulation of autophagy may also be affected with age. As previously mentioned, one example is the reduction in NRF2 activity, which may in turn result in reduced expression of antioxidant enzymes as well as core components of proteostasis machineries [97]. Another transcription factor closely connected with redoxtasis, autophagy, and longevity is FoxO. A recent study reported an age-dependent decrease in the expression of FoxO and some of its target genes in the intervertebral discs of mice. This may also be the case in humans, where decreased FoxO levels have been found in degenerating discs [165]. Only further studies can determine whether this is the case for other tissues. Whether TFEB (master regulator of lysosome- and autophagy-related gene expression) and ZKSCAN3 (the transcriptional repressor of TFEB) regulatory cascades are perturbed with age remains unclear. In any case, upregulation of NRF2, TFEB, and FoxO activity has been associated with antiageing phenotypes and extended life spans (reviewed in [166]). The expression of several miRNAs that regulate autophagy is altered during physiological ageing. For instance, miR-34 is upregulated in *C. elegans* with age and inhibits the expression of the autophagy gene ATG9A in vitro [167]. Due to the conservation of miR-34 in different organisms, it is conceivable that such an effect also occurs in mammals.

4.3. CMA Decline in Ageing. Reduced CMA activity has been observed in aged human senescent fibroblasts and lysosomes isolated from old rats [137, 168]. Both substrate binding to the lysosomal membrane and transport into lysosomes decline with age due to a progressive age-related decrease in LAMP2A levels [168]. This is not due to reduced transcription of LAMP2A, but rather the result of (1) altered mobilisation of lysosomal luminal LAMP2A to the membrane upon activation of CMA and (2) replacement of its tightly regulated cleavage at the lysosomal membrane by a less regulated LAMP2A degradation in the lumen [169]. Although the cytosolic levels and activity of HSC70 remained unchanged with age, levels of lysHSC70 were increased in the oldest rats, which suggest an attempt to compensate for the reduced activity of the pathway with age [168]. The reduction of CMA activity with age probably contributes to the accumulation of oxidised proteins, which is characteristic of most tissues in old organisms. In fact, restoration of CMA through overexpression of an inducible exogenous copy of LAMP2A in the liver of aged rodents leads to reduced levels of oxidised and aggregated intracellular proteins [170].

4.4. Proteolytic System Crosstalk. Proteolytic systems (the ubiquitin-proteasome system and the various types of autophagy) are characterised by considerable crosstalk and the ability to compensate for each other. In fact, they share substrates, effectors, and even regulators. Blocking the proteasome can result in macroautophagy induction [171] and disruption of one type of autophagy can result in the activation of either the proteasome or different types of autophagy [120, 172]. It is thought that compensation between proteolytic systems may be sufficient for maintaining homeostasis under basal conditions, but not under (severe/chronic) stress conditions. In that context, it is interesting to note that dysregulation of the crosstalk between proteolytic systems with age may result in altered proteostasis. Indeed, proteasome inhibition has been shown to activate autophagy in young but not in old rats [173]. Schneider et al. found that, while other proteolytic systems compensate for CMA loss in young mice, these compensatory responses are unable to prevent proteotoxicity induced by stress (oxidative stress or lipid challenges) in old mice [174]. Either dysregulation in the insulin pathway (which may connect the proteasome and autophagy) or TFEB signalling (probably affecting different types of autophagy) with ageing may negatively impact on the crosstalk between proteolytic systems with age [173, 174].

5. Loss of Redoxtasis and Autophagy in Alzheimer's Disease

Ageing is the main risk factor for the development of a number of diseases, including neurodegenerative diseases, cardiovascular diseases, metabolic defects, and cancer. A clear example of the deleterious consequences of the already mentioned alterations in redoxtasis and autophagy with age is provided by neurodegenerative diseases, such as Alzheimer's disease (AD). AD, the most common form of dementia in the elderly, is a proteinopathy characterized by the accumulation of insoluble aggregates of amyloid β (Aβ) peptides along with

other components in senile plaques, as well as the presence of neurofibrillary tangles of hyperphosphorylated tau. Overall, AD is considered a multifactorial process in which genetic and environmental factors along with increased susceptibility to stress with age influence each other, resulting in the loss of neuronal and brain homeostasis. As discussed below, both the loss of redoxtasis and autophagy may be part of a vicious circle with a crucial role in the pathogenesis of AD.

5.1. The Role of Oxidative Stress in AD. The central nervous system (CNS) is particularly vulnerable to ROS/RNS damage as a result of a high oxygen consumption rate, the abundance of lipids, and the reduced expression of antioxidant enzymes compared with other tissues [175]. Indeed, the "oxidative stress hypothesis" for AD and other neurodegenerative diseases supports that cumulative oxidative damage over time could account for the late-life onset and the slowly progressive nature of these disorders [176].

Many studies have shown increased markers of protein oxidation/nitration (such as protein carbonyls and 3-nitrotyrosine), oxidative-modified nucleic acids (as 8-OhdG), and AGEs in the brains of subjects with conditions ranging from mild cognitive impairment to advanced AD [176, 177]. However, whether oxidative stress is a primary cause or a consequence of some other event in AD remains elusive.

Different potential sources of ROS/RNS in AD have been proposed. Several lines of evidence indicate that Aβ itself can induce oxidative stress. For instance, the insertion of Aβ into membranes results in lipid peroxidation [177], while the effect of Aβ on microglial RAGEs produces proinflammatory signals and oxidative stress [178]. In a similar manner to Aβ, many studies support the hypothesis that modified forms of tau can produce ROS [179]. For example, mice overexpressing tau (P301S), a common mutant in tauopathies, show increased levels of carbonyls and a deregulation of antioxidant enzymes prior to neurofibrillary tangle formation [180]. Another source of ROS/RNS in AD may be damaged mitochondria. There is a general reduction in the activities of electron transport chain complexes in AD, which results in impaired mitochondrial respiration and defects in energy metabolism. Indeed, morphological, biochemical, and genetic abnormalities have been widely described in mitochondrion from AD patients [181]. Aβ was reported to accumulate in mitochondrial membranes, disrupting the electron transport chain and increasing ROS production [182].

Studies of antioxidant enzymes in AD have not shown consistent data. Aksenov et al. found increased levels of oxidative stress-handling enzymes in the parietal lobes, but not in the cerebella, of AD patients. The authors suggest that region-specific differences related to the magnitude of ROS-mediated injury are likely to contribute to variable neurodegeneration in different areas of the AD brain [183]. Another study showed elevated glutathione peroxidase, glutathione reductase, and catalase activity in specific brain regions in AD compared with normal control subjects [184]. We and others have observed increased levels of NRF2 protein together with upregulation of some of its targets, such as heme oxygenase 1 (HMOX1) and NADPH quinone oxidase 1 (NQO1), in the necropsies of AD patients [97]. Although there are contradictory observations that could reflect different stages of disease progression, these results support the notion of a compensatory antioxidant upregulation in AD brains.

5.2. Oxidative Modification of Aβ and Tau Proteins. Increased oxidative stress may affect Aβ and tau metabolism and function, leading to neurotoxicity. Aβ aggregation is accelerated by AGE-mediated cross-linking [185]. In fact, AGEs result in increased levels of Aβ per se through the upregulation of the amyloid precursor protein (APP), from which Aβ originates. Interestingly, this effect is abrogated by pretreatment with N-acetyl-cysteine (NAC), which points to its dependence on ROS [186]. Multiple studies have observed the oxidation of methionine in position 35 of Aβ. However, the functional impact of this modification is debatable, with some reports claiming it is critical to Aβ-induced oxidative stress and neurotoxicity [177] and others ascribing it a neuroprotective role [187]. The previously noted reduction in MSR with age may increase the quantities of Aβ and oxidised Met35 [177].

Tau is a natively unfolded protein that can undergo several posttranslational changes in addition to phosphorylation, including o-linked glycosylation, ubiquitination, SUMOylation, nitration, glycation, acetylation, and cross-linking [179]. The exact outcome and impact of these modifications on AD pathology remain largely unknown. It has been suggested that nitration favours tau oligomerisation and aggregation [188]. Moreover, prooxidant treatment of primary cortical rat neurons has been shown to significantly increase the aberrant hyperphosphorylation of tau in a GSK3β-dependent manner [189].

5.3. The Role of Defective Autophagy in AD. The oxidative modification and aggregation of Aβ and tau may be exacerbated by, and contribute to, impaired autophagic degradation activity. Autophagy impairment plays a crucial role in the pathogenesis of AD. In fact, excessive accumulation of autophagosomes and autophagic vacuoles (AVs) has been shown in the brains of AD patients [190]. This is likely due to incomplete autophagosome-lysosome fusion and digestion, possibly combined with induction of the initial steps of the autophagic process.

The accumulation of p62 and ubiquitinated proteins in the brains of AD patients has also been reported [191], indicating defective autophagy. Both APP and tau have been shown to colocalise with p62, suggesting their potential for being sequestered in autophagosomes for degradation [96, 192].

The underlying mechanisms for the defect in the clearance autophagosomes and their content in the neurons of AD patients are not yet fully elucidated, but several possible reasons have been reported. Familial forms of AD can be caused by inactivating mutations in presenilin 1 (PSEN1) and presenilin 2 (PSEN2). Presenilins are the catalytic subunits of the γ-secretase complex. Lee et al. suggested that full-length PSEN1 functions as a chaperone necessary for the glycosylation of the V0a1 subunit of the vacuolar (H+)-ATPase. This step is critical for its

ER-to-lysosome transport. In the absence of PSEN1, the V0a1 subunit would fail to reach the lysosomes, impairing lysosomal acidification and, consequently, the proper function of this organelle [193]. However, a recent report revisited this issue, not finding lysosomal acidification impairment in cells lacking PSEN1 or PSEN2 [194]. Genetic studies have also identified several loci associated with AD risk, including the phosphatidylinositol-binding clathrin assembly protein (PICALM) [195]. This protein is implicated in the endocytosis of SNARE proteins, necessary for fusion of autophagosomes with lysosomes. Reduced function of PICALM has been reported in AD, based on the finding of reduced full-length and increased cleaved protein levels [196]. In fact, Moreau et al. showed modulation of autophagy-dependent tau clearance by PICALM [197]. Disorganisation of the microtubule cytoskeleton due to tau hyperphosphorylation can also prevent the transport of AVs to lysosomes, further aggravating this phenotype [198, 199].

Although AVs are a major reservoir of intracellular Aβ in the brain [200], the interplay between autophagy and Aβ is complex. On the one hand, Aβ may be degraded by autophagy, as autophagy induction has been shown to reduce its levels [201]. However, Yu et al. reported that autophagosomes may be sites of Aβ production, as they detected Aβ generation-related enzymes (such as PSEN1 and nicastrin) inside these compartments [200]. Indeed, autophagy impairment has been associated with reduced extracellular Aβ deposition and plaque formation, which would hypothetically result in more intracellular and possibly toxic accumulation of the peptide [96, 202]. Overall, these studies point to the existence of more than one deficit in the autophagy pathway in AD patients. This may favour the accumulation of not only aggregated proteins but also damaged organelles and lipofuscin, which, in turn, may result in increased oxidative stress.

Moreover, different Aβ and tau modifications may alter its autophagic clearance. Interestingly, a recent report by Caballero and coworkers showed a complex interplay between different mutations and posttranslational modifications of tau and selective forms of autophagy. For instance, the A152T tau mutation, associated with higher risk of AD, disrupts its degradation by endosomal microautophagy and is rerouted towards macroautophagy degradation. Moreover, a phosphorylation mimetic of tau in the microtubule-binding domain allows tau binding to lysosomes, but its translocation is disrupted. On the other hand, mimicking phosphorylation on the flanking domains results in impaired tau binding to lysosomes. Interestingly, cells expressing either of the different tau forms analysed in this study were unable to upregulate autophagic pathways in response to oxidative stress, which reduced cell viability [203]. Future studies may clarify the impact of different Aβ modifications on autophagic degradation.

Although one study observed decreased levels of BECLIN1 in AD cortex compared to control subjects [204], we and others have found upregulation of a number of autophagy-related genes in the brains of AD patients [96, 150]. These results are opposite to what is found in normal ageing—where decreased transcription of autophagy genes has been reported, pointing to a compensatory upregulation of autophagy. In the same line of evidence, we observed increased NRF2 and its target p62 in APP- and tau-expressing neurons in AD samples [96]. NRF2-deficiency has also been shown to result in increased oxidative stress and aggravated proteinopathy in different mouse models of AD [97, 205]. The lysosomal protease cathepsin D also accumulates in AD brains compared to age-matched nondemented control brains [206]. Altogether, these data may be interpreted as the unsuccessful attempt of the diseased brain to recover homeostasis.

6. Conclusions

While the ultimate causes of ageing are complex and multifaceted, knowledge of the cellular, biochemical, and genetic changes that accompany ageing continues to grow. There is strong correlative evidence that implicates the loss of redoxtasis and proteostasis in the process of ageing and disease development. Future research should provide a better understanding of the causal relationships between these processes, which will be crucial in prolonging life span and health span and in providing new powerful tools for the development of therapeutic approaches to a wide range of pathologies.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Autophagy Modulation in Cancer: Current Knowledge on Action and Therapy

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In the last two decades, accumulating evidence pointed to the importance of autophagy in various human diseases. As an essential evolutionary catabolic process of cytoplasmatic component digestion, it is generally believed that modulating autophagic activity, through targeting specific regulatory actors in the core autophagy machinery, may impact disease processes. Both autophagy upregulation and downregulation have been found in cancers, suggesting its dual oncogenic and tumor suppressor properties during malignant transformation. Identification of the key autophagy targets is essential for the development of new therapeutic agents. Despite this great potential, no therapies are currently available that specifically focus on autophagy modulation. Although drugs like rapamycin, chloroquine, hydroxychloroquine, and others act as autophagy modulators, they were not originally developed for this purpose. Thus, autophagy may represent a new and promising pharmacologic target for future drug development and therapeutic applications in human diseases. Here, we summarize our current knowledge in regard to the interplay between autophagy and malignancy in the most significant tumor types: pancreatic, breast, hepatocellular, colorectal, and lung cancer, which have been studied in respect to autophagy manipulation as a promising therapeutic strategy. Finally, we present an overview of the most recent advances in therapeutic strategies involving autophagy modulators in cancer.

1. Introduction

Autophagy is a cellular degradation or "self-eating" pathway highly conserved throughout all life kingdoms [1]. This quality control mechanism is responsible for the degradation of protein aggregates as well as excessive or damaged organelles whose disintegrated components are later reused during the biosynthesis of new macromolecules [2, 3]. Autophagy plays an important role in maintaining cellular homeostasis and is therefore constitutively active at a basal level in most cell types. However, during different stress conditions, such as those induced by nutrient starvation, organelle damage, accumulation of abnormal proteins, or during development and cell differentiation [4], autophagy is additionally enhanced to meet the cellular needs. This multistep and fine-tuned process is regulated by autophagy- (ATG-) related proteins originally discovered in autophagy-defective yeast mutants [5].

There are three known subtypes of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). The first type, macroautophagy, is the main autophagy pathway, so the term "autophagy" usually indicates macroautophagy unless otherwise specified. During macroautophagy, a double-membrane structure, phagophore, is formed, which in a selective or nonselective manner engulfs the cytoplasmic cargo destined for degradation. Once the phagophore takes form, it gradually matures and seals, building a closed autophagosome that finally fuses with the lysosome [6] in order to degrade the autophagosome-trapped cargo. Lastly, degradation products are recycled

through the cellular anabolic reactions. In contrast, during microautophagy, the lysosomal membrane itself invaginates the cytoplasmic cargo, which is degraded in the lysosomal lumen [7]. In the third type of autophagy, CMA, the chaperone heat shock cognate protein of 70 kDa (HSC70) recognizes soluble cytosolic target proteins containing KFERQ or KFERQ-like sequence motifs, whereupon the target proteins are delivered to the lysosomal lumen through specific interaction between the HSC70 protein complex and the lysosome-associated membrane glycoprotein type 2A (LAMP2A) [8].

Originally, autophagy was thought to be an entirely nonselective process, but current knowledge demonstrates that it is also decidedly selective and that selectivity is mediated by the specific cargo-receptor proteins [9].

2. Signaling Pathways Regulating Autophagy

There are at least two major autophagy regulating pathways, ATG5/7-dependent and ATG5/7-independent [10] that were discovered subsequently. Conventional ATG5/7-dependent autophagy is initiated by the Unc-51-like kinase (ULK) complex consisting of several proteins: ULK1/2 (mammalian orthologs of yeast ATG1), FIP200 (FAK-family interacting protein of 200 kDa), ATG13, and ATG101 [11]. Under nonstressed conditions, the mammalian target of rapamycin complex 1 (mTORC1) phosphorylates ULK1/2 thereby inactivating the ULK complex [12]. In contrast, nutrient-sensitive mTORC1 is suppressed under nutrient-limited circumstances, so the ULK complex consequently remains dephosphorylated, hence activated [13]. Once activated, the ULK complex translocates to the phagophore, where it activates the class III phosphatidylinositol 3-kinase (PI3K) complex composed of VPS34 (phosphatidylinositol 3-kinase Vps34), Beclin1, VPS15, and ATG14 proteins [14]. These events lead to autophagosome formation following the extension and closure of the mature autophagosome. Two ubiquitin-like conjugation systems, ATG5-ATG12 and the microtubule-associated protein 1 light chain 3 (LC3) system, are leading regulators of the elongation and closure of the autophagosomal membrane [15–17]. In the ATG5-ATG12 pathway, ATG7 (E1-like enzyme) activates ATG12 that is transferred to ATG10 (E2-like enzyme) to finally conjugate with ATG5. This ATG5-ATG12 complex additionally interacts noncovalently with ATG16L forming a large multimeric (E3-like) complex. The tripartite complex has a function to conjugate LC3 (Atg8 in yeast) to phosphatidylethanolamine (PE) in order to be loaded as a LC3-PE conjugate, known as LC3-II, into the phagophore [18–21]. In research, this lipidated LC3-II protein is often used as a marker for monitoring autophagy progression, since it localizes to both the inner and outer membranes of phagophores and autophagosomes [22, 23].

The final step in the degradation process is the fusion of autophagosomes with lysosomes. This process is mediated by three sets of protein families: the Rab GTPases (in autophagy Rab GTPase is Rab7 protein [24, 25]), HOPS—the homotypic fusion and protein sorting-tethering complex [26], and SNAREs—the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors. Upon starvation in mammals, three SNARE proteins, including syntaxin 17 (STX17), synaptosomal-associated protein 29 (SNAP29), and vesicle-associated membrane protein 8 (VAMP8), mediate autophagosome-lysosome fusion [27, 28] (Figure 1).

ATG5/7-independent autophagy was discovered in 2009 by Nishida et al. [10]. They named it an "alternative autophagy," since ATG5 and ATG7 were until then considered crucial for mammalian autophagy [10, 29, 30]. Their main observation was that etoposide treatment of the ATG5-deficient MEFs induces autophagy to the same level as in the wild-type cells. Further, it was explained that the ULK1 complex, Beclin1, and PI3K also play a pivotal role as in conventional autophagy. In addition, they demonstrated that upon silencing the ATG5-ATG12 pathway, this did not affect the alternative autophagy, where in turn conventional lipidation of LC3 was replaced with Rab9 activity to control the extension of the phagophores [10]. Therefore, Rab9, which normally mediates transport of proteins from the late endosome to the trans-Golgi membrane [31, 32], was proposed to act in the extension and closure of phagophores in the alternative autophagy that matches the role of ATG5/ATG7/LC3 in the conventional autophagy [3]. Unlike the multiple origin of phagophores in the ATG5/7-dependent autophagy [33, 34], in an alternative autophagy trans-Golgi cisternae seem to be the only membrane source [10].

3. Autophagy Modulation as a Promising Therapeutic Target

Autophagy impairments are root causes of numerous diseases such as cancer, neurodegenerative disorders (Alzheimer disease, Parkinson disease), infectious and inflammatory diseases (Crohn's disease), diabetes, obesity, and cardiovascular and muscular diseases [35]. Therefore, the number of studies focusing on the autophagy modulation as a perspective and promising therapeutic target is constantly increasing. Some autophagy modulators, like rapamycin and its water-soluble derivatives (temsirolimus and everolimus), are already being used in cancer therapies. In 2007 and 2009, respectively, temsirolimus and everolimus were approved by the Food and Drug Administration (FDA) for the treatment of advanced renal carcinoma [36, 37]. In 2011, the FDA also approved everolimus for patients with progressive neuroendocrine tumors of pancreatic origin (PNET) [38] and for the treatment of advanced hormone receptor-positive and HER2-negative breast cancers in combination with exemestane [39]. Moreover, in 2012, the European Union approved the use of temsirolimus monotherapy for the treatment of relapsed and/or refractory mantle cell lymphoma [40].

Galluzzi summarized specific autophagy modulators and their current status in the preclinical studies and clinical trials [41]. Here, we present a summarized overview of the most prominent autophagy modulators (Figure 1).

Autophagy activators can be divided into several groups: starvation inducers, endoplasmic reticulum stress inducers, rapamycin and its derivatives, small molecule enhancers of rapamycin, trehalose, inositol monophosphatase inhibitors (IMPase), class I PI3K inhibitors, and other activators [42].

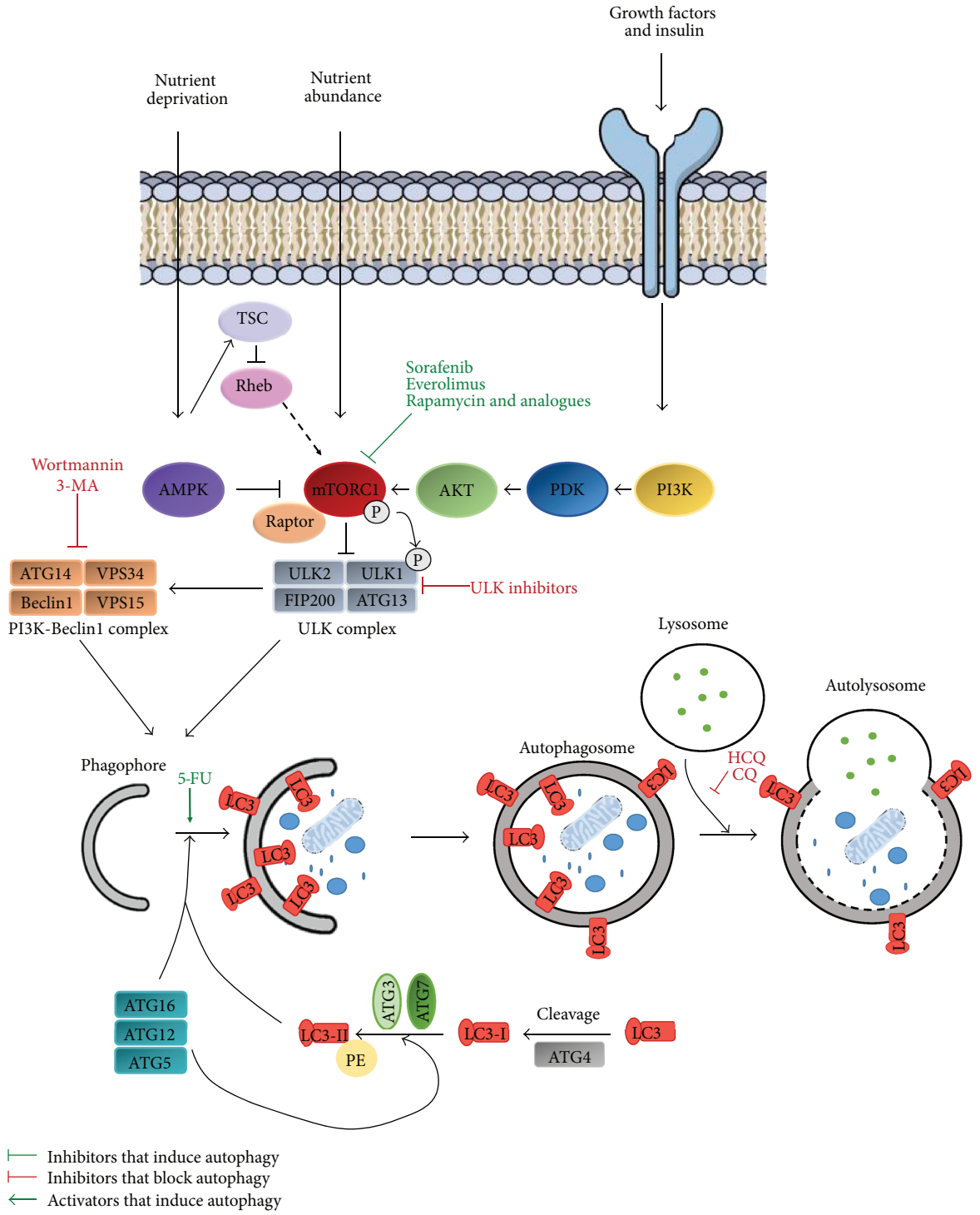

Figure 1: Schematic overview of the autophagy pathway and target points of its modulators. Activation of AMPK and inhibition of mTORC, upon nutrient deprivation, lead to ULK complex activation. Subsequently, ULK complex phosphorylates Beclin1, causing VPS34 activation and phagophore formation. Functional ULK complex consists of ULK1, ULK2, FIP200, and ATG13. VPS34, a regulatory subunit, VPS15, and Beclin1 associate with regulatory factor ATG14 forming functional PI3K-Beclin1 complex. Activation of AMPK inhibits the mTORC complex through the TSC/Rheb pathway. Multiple ATG proteins constitute two ubiquitin-like conjugation systems and mediate the generation of lipidated LC3 proteins, which direct LC3 incorporation into the phagophore membrane. Finally, an elongated phagophore closes, forming autophagosome, which then fuses with lysosome, leading to cargo degradation and nutrient recycling. Current approaches of autophagy modulation are targeting various autophagy steps: activation of autophagy by mTOR complex blockage with sorafenib, everolimus, rapamycin, and its analogues; inhibition of autophagy through inhibition of ULK complex by ULK inhibitors; inhibition of PI3K complex with 3-MA or wortmannin; and activation of autophagy through autophagosome formation induction with 5-FU and autophagosome-lysosome fusion block with HCQ and CQ.

Many of them are in preclinical studies or clinical trials, and several have already been approved for clinical use. The mTORC1 inhibitors, everolimus, and temsirolimus have been approved and are currently being used in cancer therapies, while rapamycin (sirolimus) is used in coronary stents and in rare pulmonary diseases [41, 43, 44]. Drugs such as metformin (for type 2 diabetes treatment) and retinoic acid are also approved for cancer treatment although their modes of action are still unclear [45].

Autophagy inhibitors include PI3K inhibitors, cycloheximide, vacuolar-type H(+)-ATPase inhibitors, lysosomal lumen alkalizers, and acid protease inhibitors [42]. Chloroquine (CQ) and hydroxychloroquine (HCQ) are both lysosomal inhibitors previously used for the prevention and treatment of some types of malaria [46]. Another unconventional drug, azithromycin, a macrolide antibiotic often used for treatment of multiple bacterial infections, was discovered as an autophagy inhibitor after usage in cystic fibrosis patients as an anti-inflammatory drug. Azithromycin prevents lysosomal acidification thereby blocking autophagic degradation [47]. Most of the autophagy inhibitors are still in the preclinical development stages, so we can expect their increased usage in treatment therapies in the upcoming years.

The most common approach in modulating autophagy is the targeting of two different autophagy regulation pathways: 5′ adenosine monophosphate-activated protein kinase (AMPK) and mTOR pathways [48]. The AMPK is a cellular energy sensor that is activated during stress or under scarce glucose conditions [49]. Activated AMPK induces autophagy through different pathways; hence, it is a major regulator of autophagy. This is achieved through AMPK phosphorylation of the ULK1 and TSC1/TSC2 complex that consequently acts on mTORC1 activation as we described earlier [50, 51]. AMPK also plays a very important role in selective mitochondrial autophagy (mitophagy) and mitochondrial biogenesis [50]. Further, it is responsible for the activation of peroxisome (proliferator-activated) receptor gamma coactivator 1-alpha (PGC1-α) that regulates the expression of mitochondrial proteins [52, 53]. The second target, mTOR, is a cellular nutrient, energy, and oxygen sensor kinase that regulates cell growth, survival, proliferation, and autophagy in mammals [54, 55]. mTOR kinase belongs to the PI3K-related kinase family and is the main component of the two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [56]. mTORC1 blocks autophagy and controls protein synthesis, lipid biogenesis, and cell growth [48]. Regulation of cell proliferation is controlled by mTORC2 and is crucial for cell survival and maintenance of the actin cytoskeleton [48, 57]. Dysregulation of the mTOR pathway is often correlated with cancer, neurodegenerative, cardiovascular, and renal diseases, and for this reason makes it an ideal therapeutic target [58].

4. Autophagy and Cancer

According to the latest predictions of the American Cancer Society, more than 1.6 million new cancer cases are expected to be diagnosed in 2017 in the US. The predictions are really worrying and signify that every third person affected will die from one or the other type of cancer, which is confirmed by the fact that there are about 1600 cancer deaths per day in the US [59]. The reason for these alarming statistics is the fact that carcinogenesis is one of the most complex phenomenon in the evolution of multicellular organisms. Tumor complexity causing tumor heterogeneity, which is manifested not only in different tumor types but also in the cells within an individual tumor, can enormously vary. Amazingly, every single cell within the tumor has the ability to change as the tumor progresses over time, making cancer incredibly difficult to treat. In the last two decades, we have witnessed unimaginable scientific advances in the field of molecular mechanisms underlying autophagy and have learned the importance of autophagy in physiology and disease development. Even in physiological conditions, a low level of basal autophagy is constitutively present which acts as a control mechanism of the cell and provides proper quality and quantity control of the intracellular components. The identification of the Atg genes and their products, discoveries of the molecular mechanisms [60], and the numerous loss-of-function studies on different model organisms have enabled us to understand the role of autophagy and its role in the development, differentiation [4, 61], metabolism [62], immunity [63], and aging [64].

Considering the fact that autophagy is implicated in many different cellular processes, and also keeping in mind the complexity of the molecular mechanisms of tumor initiation and development, it is not surprising that the disturbance of autophagy was found to be one of the possible causes of tumor formation and progression. Indeed, cancer was the first disease connected with disturbed autophagy [65] as well as the first for which clinical trials in humans were performed [66].

Because of the sensitive multistepped nature and complex regulatory processes, autophagy can be disrupted at any stage, which can lead to the development of a whole range of pathologies [67–69]. Therefore, targeting autophagy could be a potential strategy for the treatment of multiple diseases. Considering the dual role of autophagy in cytoprotection and cell death, it is vital to study how autophagy affects a particular type of disease in order to change its modulation in the right direction for the creation of a successful therapy.

4.1. Autophagy as a Tumor-Suppressor Mechanism. As mentioned above, autophagy plays a complex dual role in tumorigenesis and is consequently the reason why development of the autophagy-based cancer therapy is so demanding. The preliminary molecular mechanisms of tumor initiation and progression are much more complex than the mechanism of the actual disease development. The basis for any malignant cell transformation is the activation of a protooncogene or the inactivation of tumor suppressor genes. However, a large number of studies confirm that cancer cells also have altered core autophagy regulators, where either their expression levels or genetic information is altered. Knowing this, autophagy could behave similarly as a tumor suppressor (acting to prevent tumor initiation) or as a tumor promoter to ensure tumor longevity via apoptosis inhibition.

The first studies in the 1990s pointed to the relationship between autophagy and tumorigenesis and showed that about 50% of prostate, breast, and ovarian cancers have an absence of one Beclin1 allele [70–72] that codes for Beclin1, a key component in the autophagosome nucleation. Due to haploinsufficiency of the tumor suppressor Beclin1 gene, the level of Beclin1 is reduced compared to the healthy tissue, which consequently suppresses autophagy and causes cancer progression. So far, reduced Beclin1 expression has been confirmed in numerous cancers including cervical squamous cell carcinomas [73], hepatocellular carcinomas [74], osteosarcomas [75], and glioblastomas [76]. Interestingly, some studies have shown that Beclin1 gene expression is increased in stage IIIB colon cancer [77] or in non-Hodgkin lymphomas [78], which stresses the additional protooncogenic role of Beclin1.

Heterozygous deletion of several other core autophagy genes is reported to promote a tumor-suppressor role of autophagy in cancer. Monoallelic deficiency of UV radiation resistance-associated gene (UVRAG), a positive Beclin1/PI(3)K complex regulator, inhibits autophagy and contributes to the emergence of human colon [79] and gastric cancers [80]. Another UVRAG-Beclin1 complex interactor, Bax-interacting factor-1 (Bif-1), was found deleted in gastric and prostate cancers [81]. Since the core signaling pathway of the autophagosome formation is a cascade of amino acids, TORC1-ULK1-VPS34-Beclin1, nutrient starvation, and energy deficiency, common to many developing tumors, lead to mTORC1 inhibition and reactivation of ULK1 kinase activity. This activation phosphorylates Beclin1 on Ser14 and initiates the proautophagy VPS34 complexes to promote autophagy induction and maturation. Tang et al. have stressed that ULK1 can be used as a novel prognostic biomarker for breast cancer after they have found that decreased ULK1 expression is associated with cancer progression [82]. In contrast, an increased ULK1 expression observed in esophageal squamous cell carcinoma [83], hepatocellular carcinomas [84], nasopharyngeal carcinoma [85], and the latest in human gastric cancer [86] is a further indication of the dual role of autophagy as both a tumor suppressor and tumor promoter in cancer. Taking together, these data reveal potential strategic goals in cancer therapy.

4.2. Autophagy as a Tumor-Promoter Mechanism. While autophagy has a tumor-suppressing role in the early stage of carcinogenesis, in advanced cancers, it often acts as a tumor survival or even tumor promoter mechanism. This is mostly due to the fact that tumor cells are resistant to extremely stressful conditions, that is, nutrient and oxygen deprivation, within the tumor tissue. These conditions are even more rigorous in the central part of the solid tumors where the autophagy level is significantly higher than on the periphery [87]. This suggests that autophagy in some tumors also acts as an adaptive mechanism enabling their advancement in the absence of the key survival factors. Yang et al. found that an increased autophagy level in mouse pancreatic cancer led to tumor regression and prolonged lifespan [88]. Another support of the theory comes from studies where the knockouts of core autophagy proteins, ATG5, ATG7, or FIP200, were analyzed. Wei et al. analyzed and reported that with the removal of FIP200 in human breast cancer mouse models, tumor initiation and progression was suppressed [89]. The analysis of multiple cancers showed the overexpression of ATG5 in gastric [90] and prostate [91] cancers while overexpression of ATG7 was seen in bladder cancer [92]. In contrast, mice with systemic mosaic deletion of Atg5 or Atg7 developed benign liver adenomas that do not progress to adenocarcinoma or metastasize [93]. Taken together, these results demonstrate the involvement of core autophagy proteins in tumor development and progression.

In conclusion, depending on the type of tumor and its developmental stage, activation or inactivation of autophagy can contribute differently to tumorigenesis. Reduced autophagy can contribute to tumor progression, whereas increased autophagy may be a mechanism for tumor survival under hypoxic, metabolic, or therapeutic stress conditions. Thus, the modulation of the autophagy process is a promising, but complex, therapeutic strategy for the enhancement of anticancer treatments. A better understanding of the autophagy in tumor models is crucial in identifying new and effective therapeutic strategies for cancer treatment. Next, we summarize the preclinical and clinical usage of autophagy modulators in common cancer types.

Here, we outline a brief overview of current knowledge on modifications of the core autophagy machinery in pancreatic, breast, hepatocellular, colorectal, and lung cancers that represent a promising strategy for the future of drug development. Currently, these tumors represent an example of the successful application of autophagy modulation in preclinical models, which proved to be valuable for novel clinical trials. The chosen cancer types represent an example where the dual role of autophagy, both tumor promoter and tumor suppressor, has been established (Table 1). Moreover, depending on the autophagy role in cancer development and progression, specific preclinical tumor models have been designed specifically aimed at activating or to inhibiting autophagy. Most recent studies on autophagy inhibition have reported on the use of late-stage autophagy inhibitors, CQ or HCQ, which effectively inhibit autophagosome-lysosome fusion. However, their usage as autophagy inhibitors in cancer treatment is quite controversial. Current data proposes that the inhibition of late autophagy by CQ or HCQ might not be the only mechanism of their action in cancer [94–97]. Hence, they can affect tumor cell survivability through the inhibition of immune cell action against tumor cells [97] or influence the permeabilization of the lysosomal membrane thus affecting apoptosis [95]. CQ is often cytotoxic at high doses and can promote cell cycle arrest [96] or DNA damage that induces cancerogenesis [94]. In addition to studies listed in Table 1, results from preclinical studies and early-stage clinical trials with HCQ and CQ in cancer treatments have been reported for some other types of cancers.

According to http://clinicaltrials.gov, there are several ongoing phase I/II trials evaluating the combination of HCQ or CQ with chemotherapeutic agents in patients with multiple myeloma, brain, kidney, prostate, or lung cancer.

Table 1: Autophagy modulators in preclinical studies and clinical trials for cancer therapy.

	Cancer type	Autophagy modulators	Mode of action	Model tested/clinical trial phase	Reference of study/trial reference at ClinicalTrials.gov
Pancreas cancers	PDAC	CQ	Lysosomal inhibitor	Pancreatic cancer xenografts and mouse models	[100]
	PDAC	HCQ	Lysosomal inhibitor	PDAC mouse model	[101]
	Stage IIb or III pancreatic adenocarcinoma	HCQ + gemcitabine	Lysosomal inhibitor + inhibitor of DNA synthesis	Phase I/II	NCT01128296
	Advanced and metastatic pancreatic adenocarcinoma	HCQ + gemcitabine/ abraxane	Lysosomal inhibitor + inhibitor of DNA synthesis/ cell division inhibitor	Phase I/II	NCT01506973
	Metastatic pancreatic adenocarcinoma	HCQ	Lysosomal inhibitor	Phase II	[102]/NCT01273805
	Resectable pancreatic cancer	HCQ + capecitabine	Lysosomal inhibitor + inhibitor of DNA synthesis	Phase II	NCT01494155
	Resectable pancreatic adenocarcinoma	HCQ + gemcitabine + nab-paclitaxel	Lysosomal inhibitor + inhibitor of DNA synthesis + cell division inhibitor	Phase II	NCT01978184
	Resectable pancreatic adenocarcinoma	Gemcitabine, nab-paclitaxel, HCQ +/− avelumab	Inhibitor of DNA synthesis + cell division inhibitor + lysosomal inhibitor +/− T-cell activator	Phase II	NCT03344172
Breast cancers	Metastatic breast cancer	HCQ + ixabepilone	Lysosomal inhibitor + cell division inhibitor	Phase I/II	NCT00765765
	Advanced or metastatic breast cancer	CQ + taxane	Lysosomal inhibitor + cell division inhibitor	Phase II	NCT01446016
	Ductal carcinoma in situ	CQ	Lysosomal inhibitor	Phase I/II	NCT01023477
	Primary invasive breast cancer	CQ	Lysosomal inhibitor	Phase II	NCT02333890
Hepatocellular cancers	HCC after liver transplantation	Sirolimus	mTORC1 inhibitor	Phase II/III	[125]/NCT00328770
	Advanced HCC	Sirolimus	mTORC1 inhibitor	Phase II	[126]/NCT01079767
	HCC	RAD001 (everolimus)	mTORC1 inhibitor	Xenograft models of human HCC	[127]
	Advanced HCC	RAD001 (everolimus)	mTORC1 inhibitor	Phase III	[128]/NCT01035229
	HCC	RAD001 (everolimus) + BEZ235	mTORC1 inhibitor + new-generation mTORC1 (mTORC2) inhibitor	Human HCC cell lines and HCC mouse model	[130]
	HCC	RAD001 (everolimus) + SBI-0206965	mTORC1 inhibitor + ULK1 inhibitor	Different human cancer cell lines	[129]
	Advanced HCC	Sorafenib	mTORC1 inhibitor	Phase III	[132]/NCT00105443
	Advanced HCC	Sorafenib	mTORC1 inhibitor	Phase III	[133]/NCT00492752
	HCC	Sorafenib and SC-59	mTORC1 inhibitors	Human HCC cell lines and HCC mouse models	[134]

Table 1: Continued.

	Cancer type	Autophagy modulators	Mode of action	Model tested/clinical trial phase	Reference of study/trial reference at ClinicalTrials.gov
	HCC	Sorafenib + pemetrexed	mTORC1 inhibitors	Different human cancer cell lines and mouse models	[135]
	Advanced HCC	Sorafenib + HCQ	mTORC1 inhibitor + lysosomal inhibitor	Phase II	NCT03037437
	Unresectable HCC	HCQ	Lysosomal inhibitor	Phase I/II	NCT02013778
Colorectal cancers	CRC	CQ + 5-FU	Lysosomal inhibitor + inhibitor of DNA synthesis	CRC cell lines	[142, 150]
	CRC	3-MA + 5-FU	Lysosomal inhibitor + inhibitor of DNA synthesis	CRC cell lines	[77]
	CRC	CQ + 5-FU	Lysosomal inhibitor + inhibitor of DNA synthesis	CRC cell lines + mouse models	[150]
	CRC	CQ + vorinostat	Lysosomal inhibitor + histone deacetylase inhibitor	CRC cell lines + mouse models	[151]
	CRC	CQ + bortezomib	Lysosomal inhibitor + proteasomal inhibitor	CRC cell lines + mouse models	[152]
Lung cancers	NSCLC	CQ + erlotinib	Lysosomal inhibitor + EGFR-TKI	NSCLC cell lines	[157]
	NSCLC	CQ + gefitinib + cisplatin	Lysosomal inhibitor + EGFR-TKI + inhibitor of DNA synthesis	NSCLC cell lines	[158]
	NSCLC	CQ + SIRPαD1-Fc	Lysosomal inhibitor + cell division inhibitor	NSCLC cell lines	[161]
	Advanced NSCLC	HCQ	Lysosomal inhibitor	Phase I	[162]/NCT01026844
	Advanced NSCLC	HCQ + erlotinib	Lysosomal inhibitor + EGFR-TKI	Phase I	[162]/NCT01026844
	Advanced NSCLC	HCQ + erlotinib	Lysosomal inhibitor + EGFR-TKI	Phase II	NCT00977470

PDAC: pancreatic ductal adenocarcinoma; HCC: hepatocellular carcinoma; CRC: colorectal cancer; NSCLC: non-small cell lung cancer; CQ: chloroquine; HCQ: hydroxychloroquine; 5-FU: 5-fluorouracil; 3-MA: 3-methyladenine; SIRPαD1-Fc: signal regulatory protein α D1-Fc; mTORC: mammalian target of rapamycin complex; ULK1: Unc-51-like kinase 1; EGFR-TKI: epidermal growth factor receptor-tyrosine kinase inhibitor.

4.3. Pancreatic Cancer. Pancreatic cancer is the ninth leading cause of cancer deaths worldwide [59]. The most frequent form of pancreatic cancer (almost 85%) are pancreatic ductal adenocarcinomas (PDACs) which are one of the most lethal cancers worldwide [59]. The five-year survival rate is about 4% due to the fact that most PDAC patients are diagnosed with the advanced form of the cancer that has already metastasized and displays a very weak response to currently available therapies [98, 99]. There are several studies confirming the protumorigenic role of autophagy in PDAC carcinogenesis.

Autophagy inhibition with CQ or genetic manipulations by siRNA showed a positive tumor regression response in PDAC models [88]. Activating KRAS mutations were found in over 90% of PDAC [100] that further confirms the critical role of KRAS in PDAC carcinogenesis that came from mouse models [101, 102]. Additional p53 tumor suppressor gene mutations and the loss of heterozygosity have been found in ~75% of PDAC cases that contribute to tumor progression [103]. To study the impact of autophagy deficiency on PDAC, Yang et al. generated a KRAS-driven pancreatic cancer model with conditional heterozygous deletions of p53 and Atg5 alleles. Autophagy inactivation in these mice promoted the formation of premalignant pancreatic intraepithelial neoplasia (PanIN) lesions, but defected autophagy simultaneously prevented their malignant transformation to PDAC [104]. A similar study was performed by Rosenfeldt et al. using KRAS-driven PDAC models with hemizygous Atg5/Atg7 deletion and with and without the p53 mutation [105]. Mice with normal p53 expression and lower ATG5/ATG7 expression accumulated PanIN lesions that did not progress to high-grade PanIN and PDAC indicating the tumor-promoting role of autophagy in this model. In contrast, in the model without p53 and with partial Atg5/Atg7 deletion, which reduces autophagy, tumor formation was shown to be accelerated probably due to the absence of both copies of p53. The authors concluded that in the p53-mutant model, autophagy is not actively promoted. However, the question that remains unanswered is why alterations in the p53-mutant model modify the role of autophagy in PDAC carcinogenesis and tumor progression? Interestingly, although the last mentioned model showed promising results, there is no clinical trial designed to investigate the expression status of p53 and Kras genes together [105] while presently there are six clinical trials that investigate the effect of HCQ on PDAC. The first one, "Study of Pre-surgery Gemcitabine + Hydroxychloroquine (GcHc) in Stage IIb or III Adenocarcinoma of the Pancreas," examined the effect of p53 mutation status on disease-free period and their overall survivability (NCT01128296, Table 1). The results confirm the data from previous preclinical studies where p53 mutations and autophagy inactivation contributed to poor prognosis of PDAC patients, shortening the disease-free time. Another study is "A Phase I/II Pharmacodynamic Study of Hydroxychloroquine in Combination with Gemcitabine/Abraxane to Inhibit Autophagy in Pancreatic Cancer," which is currently in the recruiting phase. The endpoints of this trial will be directed to the pharmacokinetics of HCQ considering KRAS genetic status (NCT01506973, Table 1). Although it is expected that most patients with the p53 gene mutation or heterozygous deletion will also have activating KRAS mutations (known presence in more than 90% PDAC patients), the clinical trial is not examining the relationship between the expression status of p53 and KRAS. Therefore, future clinical trials should investigate the p53-KRAS interplay to ensure a proper therapeutic target and strategy in the PDAC treatment development.

Current clinical trials have shown that autophagy inhibition by HCQ as a monotherapy is not sufficient [106]. However, five clinical trials where the combination of autophagy inhibition with DNA synthesis (gemcitabine and capecitabine) or cell division (abraxane) inhibitors are used are still in progress and the results are highly anticipated (NCT01128296, NCT01506973, NCT01494155, NCT01978184, and NCT03344172; Table 1). One possible explanation as to why the combined therapies work better than HCQ monotherapy is because the autophagy could be required to degrade harmful material generated as a result of chemotherapeutic drug insults to the cancer cells. The other reason could be because the inhibition of a single possible pathway is simply not sufficient.

4.4. Breast Cancer. The first evidence of how genetic inactivation of autophagy can contribute to the malignant transformation in breast cancer was made by Liang and colleagues in 1999 [107]. They showed that Beclin1 expression is frequently low in human breast epithelial carcinoma cell lines and tissues, but expressed at high levels in normal breast epithelia. Further, they noted how Beclin1 in MCF7 breast carcinoma cells has an autophagy-promoting activity. These findings suggested that the decreased expression of autophagic protein Beclin1 might contribute to the development or progression of breast cancer.

Since 2003, several studies have been performed on genetically engineered breast cancer mouse models with impaired autophagy. Qu et al. and Yue et al. have used a Beclin1 heterozygous mouse model to test whether monoallelic deletion of Beclin1 promotes breast cancer tumorigenesis [108, 109]. This further evidenced how genetic inactivation of autophagy can contribute to a malignant transformation.

A study by Wei et al. revealed that FIP200 as a potential target for cancer therapy since FIP200 ablation in mice, and consequently autophagy inhibition, suppressed mammary tumor initiation and progression [89]. p62/SQSTM1 (known as sequestosome-1, here referred to as p62) is a selective autophagy receptor that binds LC3 and recruits the selected cargo to the maturing autophagosome [110, 111]. The disruption of essential genes within the autophagy pathway, including FIP200, impairs autophagosome biogenesis at the earliest stages and leads to the accumulation of substrates such as p62. As it interacts with a number of proteins in different intracellular signaling pathways, p62 plays an important role at the crossroads of autophagy, apoptosis, and cancer [112–114]. Several studies [115, 116] showed that p62 has a role in protumorigenesis, and Mathew et al. [117] found that p62 accumulation, upon autophagy inhibition in apoptosis-deficient cells, increased tumorigenesis through

increased oxidative stress and deregulation of NF-κB signaling. Wei et al. have demonstrated that p62 knockdown or p62 deficiency in already established FIP200-null tumors dramatically reduced tumor growth [118]. Therefore, this later model demonstrated that p62 impairment and suppression of autophagy by FIP200 deletion could synergize to inhibit tumor growth, suggesting new insights for the future design of anticancer drugs.

A study from 2010 revealed a significant association between Beclin1 deletion and human epidermal growth factor receptor 2 (ErbB2) amplification [119], thus providing evidence for decreased Beclin1 expression in a particular breast cancer subtype [120]. A recently established mouse tumor model made by Lozy et al. was the first link between Beclin1 heterozygosity and ErbB2-driven mammary tumorigenesis [121]. This model showed that ErbB2-driven cancers were associated with autophagy suppression. With the proposal of a new model for the PALB2- (partner and localizer of BRCA2) associated hereditary breast cancer, Huo et al. directly demonstrated a tumor-promoting role of autophagy in breast cancer development [122]. Under normal conditions, PALB2 functions as a tumor suppressor similarly to BRCA1 and BRCA2 in maintaining the genome stability and cellular homeostasis. Due to the monoallelic deletion of Beclin1, impaired autophagy results in reduced PALB2-associated breast tumorigenesis in the wild-type p53, but not in a conditionally null background, indicating that Beclin1-related autophagy plays a protumorigenic role in the wild-type p53 PALB2-associated breast cancer, but not in the p53 null tumors.

Currently, several clinical trials are testing the therapeutic potential of different autophagy inhibitors that are used alone or in combination with chemotherapeutic agents. Phase I/II clinical trial was performed to investigate the role of autophagy inhibition on metastatic breast cancer patients using a combined treatment of HCQ and ixabepilone, a chemotherapeutic agent that stabilizes microtubules (NCT00765765, Table 1). The aim of this clinical trial was to show a decrease in tumor growth and a higher tumor response compared to ixabepilone chemotherapy alone. Unfortunately, due to slow patient accrual, the study could not be completed (the main reason for the low accrual was too stringent exclusion criteria). Two clinical trials testing CQ are currently in the recruitment phase. The first one, a phase II study (NCT01446016, Table 1), is testing the effect of CQ in combination with taxane or taxane-like chemotherapy in metastatic breast cancer patients who have previously failed anthracycline chemotherapy. The second, a phase I/II study (NCT01023477, Table 1), investigates the reduction of ductal carcinoma in situ (DCIS) after CQ administration. Yet another phase II trial examines CQ effect on breast tumor cell proliferation and apoptosis (NCT02333890, Table 1). The point of this study is to determine whether CQ will prevent breast cancer growth in patients currently not being treated with neoadjuvant chemotherapy prior to surgical intervention. To conclude, due to the high heterogeneity of this type of cancer, it might be difficult to find unique treatments, making breast cancer a good candidate for a more personalized approach.

4.5. Hepatocellular Cancer. Incidence of the liver cancer has been more than tripled from 1980 onwards. A majority of primary liver cancers (75–90%) are hepatocellular carcinomas (HCCs), a carcinoma with very high malignant potential, high recurrence rate, and poor patient prognosis. In the early stage, surgical resection or liver transplantation are proven to be successful. However, for patients at the advanced HCC stage, effective therapy is currently unavailable resulting in low overall survival rates. Autophagy plays multiple roles in maintaining liver homeostasis. In the absence of key autophagy genes, Atg5 and Atg7, nonfunctional proteins and organelles accumulate in liver cells [93]. Analysis of conditional Atg7 knockout mice revealed that these mice develop hepatomegaly and different metabolic liver disorders [30]. HCC is one of the best examples of dual autophagy role in tumors. In the early stage of HCC, during hepatocyte dysplasia, an antitumor role applies. Autophagy contributes to the preservation of the genome stability and the prevention of a malignant transformation by removing harmful mitochondria and transformed cells. A rat model study demonstrated that in the established HCC treatment with CQ, autophagy inhibition acquired a protumor role providing nutrients to HCC cells in the tumor microenvironment and promoted enhanced tumor growth [123]. The tumor-suppressing role of autophagy in HCC has been confirmed on several HCC models. One of the first evidence supporting the tumor suppressor role of autophagy in the cancer formation comes from Beclin1 knockout mice. Biallelic Beclin1 deletion reduces autophagy activity and such mice were more likely to develop cancer, including HCC [108]. In addition, the heterogenic deletion of the Beclin1 reduced autophagy, increasing cell proliferation and initiating spontaneous formations of malignant lesions [108, 109]. It is also interesting to note the correlation between the decreased expression of Beclin1 and HCC grade [74]. This unfolds the possibility to use the Beclin1 gene as a HCC prognostic biomarker [74]. Takamura et al. [93] reported that mice with systemic mosaic deletion of Atg5 exclusively develop benign liver adenomas. These tumors show disordered autophagy features such as mitochondrial swelling, p62 accumulation, oxidative stress, and genomic damage responses. Liver-specific Atg7-deficient mice also developed liver tumors that were reduced in size after p62 deletion [93]. This suggests two things, that autophagy is important for the suppression of tumorigenesis in the liver and that the accumulation of p62 caused by autophagy deficiency contributes to tumor progression.

The latest insight about the antitumor role of autophagy was given by Chen and colleagues [124] who demonstrated that the long noncoding RNAs (lncRNAs) have a regulatory role in HCC. In healthy cells, an elevated expression of the phosphatase and tensin homolog (PTEN) and its homolog PTENP1 results in the PI3K/AKT pathway inhibition. This consequently suppresses the cell proliferation and induces autophagy and apoptosis. As opposed to this, Chen and colleagues have found that the expression of PTEN and PTENP1 is downregulated in several HCC cell lines. However, they have shown that the PTENP1 activity could be restored by lncRNAs or miRNAs. The mentioned miRNAs

can also increase the expression of autophagy genes including ULK1, ATG7, or p62, trigger autophagy, and suppress HCC tumor growth. This points to the possibility of using precisely targeted RNA in HCC therapy, but probably in other tumors too.

Cancer cells use autophagy to ensure an alternative energy source for growth and survival in a stressful tumor microenvironment with high hypoxia, scarce nutrients, and very often therapeutic stress conditions. It was reported that in advanced HCC, autophagy has an oncogenic (prosurvival) role observed as an increased LC3-II expression that positively correlates with malignant progression and poor prognosis [125]. However, one should be careful when making conclusions regarding the influence of LC3-II overexpression on autophagic activity. Hence, LC3-II overexpression cannot be solely used as a marker for increased autophagic activity since LC3-II increased expression might have resulted from accumulation due to autophagy inhibition at the postlipidation stage. Specific hepatocyte Atg5 knockout mice revealed the tumor promoter role of autophagy in hepatocarcinogenesis [126]. Thus, the Atg5 ablation resulted with impaired autophagy in the liver and the development of benign hepatic tumors with no hepatocellular carcinoma. This inability to develop hepatocellular carcinoma was correlated with the induction of tumor suppressors, such as p53, p16, p21, and p27, which negatively regulated the progression of tumorigenesis when autophagy was impaired. Hence, autophagy in advanced HCC may block antitumor effects of tumor suppressors, and blocking autophagy altogether may be a promising target for the therapy of established HCC.

Since autophagy plays a dual role in the pathophysiology of malignant liver diseases, it is important to define whether a specific cancer type requires autophagy induction or inhibition to propagate. Back in 1985, Schwarze and Seglen were the first to indicate that autophagy acts to suppress liver carcinogenesis and HCC growth by limiting the cellular protein accumulation rate [65]. In 1993, Kisen et al. observed reduced autophagic activity in HCC cells and their precursors compared to normal hepatocytes [127]. This confirmed the hypothesis that reduced autophagy may be an important aspect of growth deregulation in liver cancer. Rapamycin and its derivatives act as autophagy inducers by inhibiting mTOR pathway [128]. In clinical trials, with the introduction of rapamycin, antitumor effect was shown along with improved overall survival rates in post liver transplantation patients with HCC (NCT00328770, Table 1) [129]. A similar effect was observed after using sirolimus in patients with advanced HCC who did not have a liver transplantation (NCT01079767, Table 1) [130]. This needs to be highlighted because it indicates that autophagy activation, and not only inhibition, may be beneficial in the HCC treatment. Unfortunately, everolimus, a rapamycin derivate, did not give such promising results and conflicting data have been obtained. Everolimus inhibits tumor growth in xenograft models of human hepatocellular carcinoma [131]. However, the later clinical phase III trial, EVOLVE-1, performed on patients with progressed HCC after sorafenib treatment or on patients that showed intolerance to sorafenib, did not show a significant difference in the overall survival rate when everolimus was administered (NCT01035229, Table 1) [132]. To that end, cell line experiments using a combination of different autophagic pathway targets indicate that this could be a promising therapeutic strategy in HCC treatment [133, 134]. For example, the synergistic effect of targeting mTOR with a combination of everolimus and another new-generation phosphatidylinositol 3-kinase/mTOR adenosine triphosphate-site competitive inhibitor BEZ235 or with the ULK1 inhibitor [133] suppresses proliferation of tumor cells.

Sorafenib, a multityrosine kinase inhibitor of mTOR pathway, an FDA-approved drug that induces autophagy, is also used for the advanced HCC treatment [135, 136]. Preclinical studies and several clinical trials confirm that sorafenib improves the survival of patients with advanced HCC, independently (NCT00105443, NCT00492752; Table 1) [136, 137] or in coadministration with other small-molecule drugs that inhibit HCC growth through autophagy induction [138, 139]. The problem with sorafenib-induced autophagy is a possible drug resistance in patients with HCC [140]. Therefore, further research is necessary to clarify the role of an autophagy-induced therapy approach in the treatment of HCC. Moreover, autophagy inhibition could also be a potential therapeutic strategy for HCC treatment, because autophagy is required for HCC cell survival, especially in the early stages [141]. Although there are about fifty ongoing clinical trials based on targeting autophagy for cancer treatment, only two current clinical trials are focused on autophagy inhibition using HCQ in HCC. The first one, a phase II trial, is studying if sorafenib-/HCQ-induced autophagy in HCC will have improved efficacy when compared to sorafenib treatment alone and if the addition to HCQ would lead to disease stability in patients with advanced HCC (NCT03037437, Table 1). The second clinical trial is in the recruiting phase I/II focusing on autophagy inhibition using HCQ in unresectable HCC (NCT02013778, Table 1).

4.6. Colorectal Cancer. Colorectal cancer (CRC) is the third major cause of cancer deaths in both sexes worldwide with the relative 5-year survival rate ranging around 65% after being diagnosed [59]. A complex multifactorial etiology of CRC is known, and, in recent years, autophagy has been recognized as one of the molecular mechanisms that regulate malignant transformation of CRC cells. Moreover, successful autophagy modulation, in several studies, proved to be very promising as CRC therapy.

The autophagy machinery provides multiple genes involved in the switch from normal to colorectal pathology. The first link between autophagy and CRC was discovered when autophagosomal marker, LC3-II protein, was found overexpressed in an advanced CRC compared to normal surrounding tissue [142, 143] suggesting that altered LC3 expression levels could indicate autophagy involvement in cancer. Also, low LC3 level has been linked to a better therapeutic response and prognosis in patients with advanced CRC [144, 145] suggesting a possible role of LC3 protein as a prognostic CRC marker. The prosurvival role of autophagy was suggested on the CRC cell line models after treatment with autophagy inhibitors, 3-methyladenine (3-MA) or CQ

in combination with 5-fluorouracil (5-FU) and radiation therapy [77, 146].

Current knowledge on the role of Beclin1 gene in colorectal cancer is quite controversial since different studies show the opposite roles of Beclin1 in CRC carcinogenesis. Studies are reporting that Beclin1 overexpression can support tumorigenesis [147] but may also inhibit CRC cell growth [148]. This controversy is particularly emphasized when the survival prognosis is correlated with the Beclin1 expression and CRC. Both Zhang et al. and Ahn et al. have shown high Beclin1 expression in colorectal carcinoma tissue compared to healthy mucosa [147, 149]. Additionally, Ahn et al. studied this high Beclin1 expression in respect to invasion, metastasis, and cancer stage. However, no significant association of Beclin1 expression with clinicopathologic characteristics was reported [149]. Interestingly, another study analyzing Beclin1 expression in CRC patients has shown that those patients with a high Beclin1 expression had a better chance of being disease-free and had a better overall survival rate as compared to those with lower Beclin1 expression, indicating that high Beclin1 expression could serve as a favorable prognostic marker in CRC [150]. The controversial role of Beclin1 in CRC carcinogenesis was demonstrated by the results of two contradictory studies that tried to explain the connection between Beclin1 expression with a final clinical outcome in CRC patients who received 5-FU-based adjuvant therapy after resection. The first study showed that an increased expression of BECN1 is connected with a better clinical prognosis in patients who received 5-FU chemotherapy 6 months after resection [151]. The second study reported that Beclin1 overexpression was associated with reduced survival in CRC patients treated with 5-FU indicating a role for autophagy in drug resistance [152]. Further, low Beclin1 expression in patients with advanced CRC treated with cetuximab has been connected with longer disease-free survival [144] while later studies from 2014 to 2015 suggested a low Beclin1 expression as a prognostic biomarker for poor final clinical outcome [145, 153]. With this in mind, the role of Beclin1 in CRC still represents a complex puzzle in need of more extensive research.

Alterations in other core autophagy machinery components have also been associated with CRC and its progression and may potentially be good prognostic indicators. Mutations and reduced expression of Atg5 were found in many gastrointestinal carcinomas including CRC [154], suggesting the tumor suppressor role of autophagy in CRC. Unlike Atg5, the expression of Atg10, important in the elongation of the autophagosomal membrane, was increased in CRC and associated with invasiveness and generally worse prognosis [155].

Currently, there are several preclinical studies on CRC cell lines or mouse models using autophagy inhibitor CQ in combination with other agents such as 5-FU [156], histone deacetylase inhibitor—vorinostat [157], or proteasomal inhibitor—bortezomib [158]. It was shown that more successful effects are achieved with combined therapy than with monotherapy in respect to tumor growth reduction.

To conclude, the role of autophagy in CRC is still unclear and future studies will need to consider particular carcinomas individually to develop personalized anticancer therapy, which would take into account the autophagy status and its specific molecular changes.

4.7. Lung Cancer. The highest mortality rates of all cancer types belongs to lung cancer [59]. The lack of obvious symptoms in the early stages of the disease greatly postpones the diagnosis, which unfavorably affects the outcome of the disease. There are two major subgroups of lung cancer classified according to their histological appearance, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). The NSCLC is the most common type accountable for 85% of all lung cancer diagnosis [159]. Currently, the genetically engineered mouse models whereby the NSCLC initiation and progression are driven by the oncogenic KRAS or BRAF mutations are used to study the molecular aspects of the disease [159].

Previous studies on different cancer cell lines bearing activating mutations in Ras have shown that the autophagy level is higher than in the healthy cells suggesting that these tumors are autophagy-dependent and that autophagy could serve as a potential therapeutic target in NSCLC treatment [116, 160]. When the Atg7 was deleted in the mice lungs bearing NSCLC, a suppression of the tumor cell proliferation was noticed [116, 160]. These experiments have also shown that NSCLC cells with impaired autophagy accumulate morphologically abnormal mitochondria indicating that intact mitochondrial function is important for the growth and malignancy of NSCLC. It was also shown that due to impaired respiration and oxidation of the fatty acids, these tumors have a tendency to accumulate lipids and are more prone to starvation. Similarly, the Atg7 deficiency in mice with BRAF-induced lung tumor also resulted in accumulation of dysfunctional mitochondria ultimately leading to tumor growth restriction [161]. But one interesting difference between KRAS- and BRAF-driven NSCLC was also observed that relates to the mice overall survival. BRAF mutants with Atg7 deletion tend to live longer as opposed to mice with KRAS mutation that die from pneumonia instead of cancer suggesting that autophagy deficiency might promote inflammation [162].

Understanding of the exact mechanism by which autophagy promotes the NSCLC growth and progression would open the door to prospective therapy. To this end, Strohecker et al. have shown that in Atg7-deficient KRAS- or BRAF-driven NSCLC, addition of glutamine rescues the tumor progression suggesting that autophagy provides amino acids needed to fuel tricarboxylic acid cycle (TCA) [160, 161]. Therefore, it can be concluded that autophagy promotes malignancy through maintenance of the mitochondrial function in such a manner that it ensures availability of the mitochondrial substrates crucial for preventing energy deprivation. These studies have opened up a possibility to control KRAS- or BRAF-driven NSCLC growth by specifically modulating autophagy.

In addition, Zou et al. have shown that some NSCLC cells have elevated levels of autophagy as a consequence of the treatment with EGFR tyrosine kinase inhibitors (TKI) [163]. It was speculated that the observed upregulation of autophagy could be an alternative mechanism that promotes

tumor cell survival in NSCLC cells resistant to the EGFR-TKI treatment. Therefore, targeting autophagy in combination with EGFR-TKI seemed a good treatment option that could overcome the resistance and enhance antitumor effect of these drugs. Indeed, two studies have shown that when an autophagy inhibitor CQ is used in combination with EGFR-TKI, NSCLC cells are more prone to respond to treatment either by overcoming the resistance in the wild-type EGFR NSCLC or by overcoming the antagonistic effect of EGFR-TKIs and therapeutic agents in wild-type and mutant EGFR NSCLC [163, 164].

A chemotherapeutic drug paclitaxel, also used in NSCLC treatment, was shown to be ineffective in some NSCLC. Apparently, paclitaxel induces the autophagy through decreased miR-216b levels that normaly downregulate Beclin1 activation and causes autophagy activation in paclitaxel-treated cells resulting in a decreased paclitaxel-induced cell death due to the activation of autophagic cancer cell survival [165]. Along similar lines, other preclinical studies conducted on NSCLC cells investigated the effect of the hormonally active form of vitamin D (1,25-D3) and vitamin D analogue (EB1089) in combination with radiation. It was found that EB1089 induces a novel cytostatic form of autophagy suppressing NSCLC proliferation [166]. Additionally, Zhang et al. have shown that simultaneous targeting of CD47 and autophagy in NSCLC xenograft models enhance antitumor activity through the activation of caspase-3, recruitment of macrophages, and overproduction of ROS [167].

Up until now, there have only been two clinical studies investigating the effectiveness of HCQ in combination with EGFR-TKIs in NSCLC treatment. One such study, published in 2012, was conducted to explore the safety, maximal dose tolerated, clinical response, and pharmacokinetics of HCQ with or without EGFR-TKI inhibitor, erlotinib [168]. This study has shown that HCQ is generally well tolerated and safe in NSCLC patients previously treated with EGFR-TKIs. However, low response rates observed in the study cohort were assigned to potentially ineffective doses of HCQ or by heavily pretreated patient populations (NCT01026844, Table 1) [168]. The other currently ongoing study is investigating erlotinib with or without HCQ in chemo-naive advanced NSCLC (NCT00977470, Table 1).

Overall, the clinical data on autophagy inhibition in NSCLC patients is scarce but promising, suggesting that further work is needed to fully elucidate the possibilities of autophagy inhibition especially in patients with NSCLC resistant to EGFR-TKIs.

5. Conclusions and Future Perspective

The role of autophagy in cancer is enormously complex, and our knowledge in this field is currently very limited. Deciphering the autophagy in context of tumor complexity and heterogeneity is necessary to fully understand their complex and intertwined association.

Despite the seemingly paradoxical and dual role of autophagy in the context of tumor initiation and development, studies on cellular and mouse models have confirmed that there are two main principles of autophagy actions in tumors. Autophagy-deficient mouse models demonstrate that at the beginning of tumor development, basal autophagy is generally able to inhibit tumor formation by suppressing the DNA damage and genome instability. However, in contrast to this suppressive role, autophagy facilitates tumor progression in most cancers. Does autophagy always serve as a mechanism for providing energy and nutrients in developing cancers or is this theory only limited to some cancers? Most of the current studies confirm that cancers use autophagy to obtain nutrients needed to sustain tumor growth, but the question is why autophagy suppressor-based therapies are often not effective enough? Our understanding of what autophagy specifically does at the molecular level and how it influences different tissues, tumors, and genes is currently very limited. Albeit, there is expanding knowledge from preclinical studies and clinical trials on the possible utilization of autophagy as an anticancer immunotherapy. The first results indicate that optimal combination of autophagy inducers or inhibitors with chemotherapy is going to be important approaches for even more successful therapies (summarized by Pan et al., *Oncotarget*) [169]. Additionally, do we know which type of autophagy contributes to the development of a single tumor and could selective autophagy respond to the question of unsuccessful autophagy suppressor-based therapy of cancer? To better understand the role of autophagy in tumors, additional basic research is needed in the field of molecular biology, biochemistry, and chemistry but above all in molecular oncology.

According to the latest data, 53 out of 81 international clinical studies investigating autophagy modulation as a possible target in disease therapy are focusing on cancer. Based on the fact that about 70% of clinical studies are focusing on autophagy role in cancer indicates that the potential of autophagy modulation in cancer treatment is promising. Clinical trials involving autophagy modulation in cancers have been designed to assess the effect of autophagy inhibition in combination with other conventional therapies. Only a minority of clinical trials on lung, glioblastoma, pancreatic, melanoma, breast, and prostate cancers are testing CQ/HCQ as a monotherapy. This is most likely due to the fact that we currently do not have good autophagy-specific modifiers as well as due to cancer complexity where it is almost exclusively needed to target more than one cellular pathway to generate a successful therapy. Therefore, it is important to encourage chemists and pharmacologist to design and synthesize novel and highly specific autophagy modulators and to further engage cellular and molecular biologists, computational biologists, and mathematicians in pharmaceutical industry and academia to a collaborative network.

The opened question remains, why combined therapy is almost always more effective than monotherapy and what are the consequences for using this aggressive combination? Based on preclinical animal studies, so far there are not many reported side effects of autophagy inhibition such as cancer remission, appearance of secondary tumors, metabolic disorders, or infections (except potential induction of inflammation in case of autophagy deficiency mentioned earlier).

Moreover, there is no available data considering the consequences of other molecular pathway inhibitions that are used in combined therapy. Unfortunately, we still lack the knowledge and tools to specifically activate or inactivate autophagy without disturbing other cellular processes and to specifically modulate autophagy within the tumor cells concurrently avoiding autophagy disruption in healthy cells.

A major problem with the clinical trials is how to identify patients who are most likely to benefit from autophagy-based cancer therapy. To estimate if autophagy modulation is an effective therapy, the critical point would be to measure the autophagic flux *in vivo*. Currently available biomarkers for monitoring autophagy flux in clinical trials consist of tracking the accumulation of autophagic vesicles in tumor cells as well as monitoring the status of LC3 lipidation by Western blotting or immunohistochemistry. The basic research scientists are at the moment less limited with the tools and resources necessary for the complete autophagy assessment in different settings and conditions. However, much more *in vivo* experimentation is needed for new or improved techniques, so taken together it makes future clinical applications more difficult and challenging.

What is well known is that disturbed autophagy is the basis for many diseases including cancer. To date, translating preclinical knowledge of autophagy modulation into clinic has progressed rapidly in the field of oncology. Although there are very few reported clinical results confirming the effectiveness of autophagy modulation in cancer, in several other pathologies, such as obesity and diabetes, cardiology, neurobiology, and immunology, preclinical studies give promising results. Therefore, it is a huge challenge and task for both, scientists and clinicians, to better understand the molecular mechanisms of autophagy and carcinogenesis in order to successfully translate preclinical knowledge to a clinical environment. Also, the development of new autophagic modulators, activators or inhibitors, is necessary to selectively target the newly discovered autophagic signaling molecules that represent the key players in both canonical and alternative autophagy pathways. Above all, it will be of great importance to access the individual approach to cancer and its autophagy status.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Mija Marinković and Matilda Šprung contributed equally to this work.

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Research Article

Epigallocatechin-3-Gallate (EGCG) Promotes Autophagy-Dependent Survival via Influencing the Balance of mTOR-AMPK Pathways upon Endoplasmic Reticulum Stress

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The maintenance of cellular homeostasis is largely dependent on the ability of cells to give an adequate response to various internal and external stimuli. We have recently proposed that the life-and-death decision in endoplasmic reticulum (ER) stress response is defined by a crosstalk between autophagy, apoptosis, and mTOR-AMPK pathways, where the transient switch from autophagy-dependent survival to apoptotic cell death is controlled by GADD34. The aim of the present study was to investigate the role of epigallocatechin-3-gallate (EGCG), the major polyphenol of green tea, in promoting autophagy-dependent survival and to verify the key role in connecting GADD34 with mTOR-AMPK pathways upon prolonged ER stress. Our findings, obtained by using HEK293T cells, revealed that EGCG treatment is able to extend cell viability by inducing autophagy. We confirmed that EGCG-induced autophagy is mTOR-dependent and PKA-independent; furthermore, it also required ULK1. We show that pretreatment of cells with EGCG diminishes the negative effect of GADD34 inhibition (by guanabenz or siGADD34 treatment) on autophagy. EGCG was able to delay apoptotic cell death by upregulating autophagy-dependent survival even in the absence of GADD34. Our data suggest a novel role for EGCG in promoting cell survival via shifting the balance of mTOR-AMPK pathways in ER stress.

1. Introduction

Green tea is a type of traditional Chinese tea made from *Camellia sinensis* leaves, and it has been demonstrated to possess profound biochemical and pharmacological activities, including antioxidative, anti-inflammatory, and anticarcinogenic properties [1–3]. Green tea contains several polyphenolic components, for example, catechin, epicatechin, and epigallocatechin-3-gallate (EGCG). Effects of the most abundant green tea polyphenol EGCG have been shown in various pathophysiological conditions, including insulin resistance, endothelial dysfunction, and ischemia-reperfusion injuries [4–6]. Many scientific reports proposed that green tea is able to influence several biological processes by inhibiting telomerase, mitogen-activated protein kinase (MAPK), activator protein-1, or nuclear factor- (NF-) κB [7]. It has been also shown that EGCG is able to extend longevity significantly under several stress conditions by postponing aging and age-related diseases [8–10].

Cellular homeostasis is finely controlled by an evolutionarily conserved cytoprotective cellular digestive process, called autophagy [11]. Cells have residual autophagic activity even under physiological conditions; however, the process gets more efficient during various stress events (i.e., starvation and growth factor deprivation) [12]. During autophagy, cellular components become sequestered into a double-membrane vesicle, whose contents are then delivered to and degraded by lysosomes [13, 14]. Due to the crucial role of

autophagy in maintaining cellular homeostasis, this self-eating process is precisely regulated [11]. Interestingly, an excessive level of autophagy is also known to cause cell death [15].

One of the key roles of autophagy is to maintain essential cellular activity and viability under limited nutrient availability [13]. Therefore, autophagy is tightly controlled by the two sensors of nutritional conditions, called mTOR and AMPK [16–18]. mTOR (mammalian target of rapamycin) is a serine/threonine protein kinase in the mTORC1 complex, which is the main component of the mTOR pathway [18, 19]. This complex is a master regulator by integrating inputs from external and internal signals, such as growth factors, amino acids, glucose, and energy status, to control growth and metabolism [20]. Besides mTOR, AMPK (AMP-activated protein kinase) also senses cellular energy status and has a crucial role in maintaining energy homeostasis [21]. AMPK tightly controls ATP-consuming processes, such as glycogen or protein syntheses, and it upregulates processes that yield ATP (i.e., glycolysis) [22, 23]. AMPK is able to promote self-eating by phosphorylating ULK1, one of the main inducers of autophagosome formation [17, 21]. However, ULK1 is also regulated by an mTOR-dependent inhibitory phosphorylation under nutrient-rich condition [21]. In addition, AMPK directly inhibits mTORC1 complex via phosphorylation [17, 21] indicating that a proper balance of AMPK-mTOR pathways is essential at physiological conditions.

It has been lately suggested that EGCG might induce a cytoprotective autophagy in various stress events. Treatment with EGCG promotes the formation of autophagosomes both in primary bovine endothelial and human hepatoma (HepG2) cells [24, 25]. EGCG abolishes the palmitate-induced accumulation of lipid droplets via facilitated autophagic flux [24]. Autophagy enhancement upon EGCG administration is unfavourable for hepatitis B virus replication, and hence it is considered as a potential therapeutic strategy [25]. EGCG also has neuroprotective effect by activating autophagy and inhibiting Bax and cytochrome *c* translocation in prion-protein-induced damages [26]. Although the positive role of EGCG in enhancing autophagy at various diseases has been already suggested, details of the regulatory mechanisms induced by this natural compound are yet to be revealed.

Huang et al. have suggested that EGCG upregulates AMPK activity in a dose-dependent manner, while mTOR pathway gets inhibited in hepatoma cells [27]. A docking experiment has also shown that EGCG is an ATP-competitive inhibitor of mTOR [28]. Kim et al. suggested that EGCG enhances autophagy through an AMPK-mediated mechanism [24]. Interestingly, EGCG stimulated both AMPK and ULK1, but not mTOR, indicating that the polyphenol-induced autophagy is independent from mTOR pathway [24]. These results suggest that EGCG acts as an enhancer on AMPK; however, its effect on mTOR pathway is still contradictory.

Recently, we have confirmed that activation of autophagy has a cytoprotective role upon high level of endoplasmic reticulum (ER) stress [29, 30]. This transient elevation of autophagy is characterized by downregulation of mTOR and upregulation of AMPK. Therefore, mTOR inhibitors and/or AMPK activators (such as rapamycin, resveratrol, and metyrapone) are able to postpone apoptotic cell death during excessive ER stress [29, 31]. EGCG is able to restore Ca^{2+} homeostasis suggesting its cytoprotective effect in ER stress [32]; however, the detailed mechanism of the EGCG-modulated ER stress response remains to be elucidated.

In this study, we investigate the mechanism of EGCG-dependent autophagy and its role in ER stress by using a human cell line. We propose that the cytoprotective autophagy stimulated by EGCG is regulated via both mTOR and ULK1. We also show that EGCG-induced self-eating process is independent from PKA. Here, we present that EGCG affects the balance of mTOR-AMPK, which delays apoptotic cell death by upregulating autophagy upon ER stress. Our data demonstrate a novel mechanism underlying the effect of EGCG on life-and-death decision in ER stress.

2. Materials and Methods

2.1. Materials. Thapsigargin (Sigma-Aldrich, T9033), tunicamycin (Sigma-Aldrich, T7765), rapamycin (Sigma-Aldrich, R0395), guanabenz (Sigma-Aldrich, G110), H89 (Adipogen, AG-CR1-0002), and epigallocatechin gallate (Sigma-Aldrich, E4143) were purchased. All other chemicals were of reagent grade.

2.2. Cell Culture and Maintenance. A human embryonic kidney cell line (HEK293T, ATCC, and CRL-3216) was used as a model system. It was maintained in DMEM (Life Technologies, 41965039) medium supplemented with 10% fetal bovine serum (Life Technologies, 10500064) and 1% antibiotics/antimycotics (Life Technologies, 15240062). Culture dishes and cell treatment plates were kept in a humidified incubator at 37°C in 95% air and 5% CO_2.

2.3. SDS-PAGE and Western Blot Analysis. Cells were harvested and lysed with 20 mM Tris, 135 mM NaCl, 10% glycerol, and 1% NP40, pH 6.8. Protein content of cell lysates was measured using Pierce BCA Protein Assay (Thermo Scientific, 23225), and equal amounts of proteins were used in the analysis. SDS-PAGE was done by using Hoefer miniVE (Amersham). Proteins were transferred onto Millipore 0.45 μM PVDF membrane. Immunoblotting was performed using TBS Tween (0.1%), containing 5% nonfat dry milk, 1% bovine serum albumin (Sigma-Aldrich, A9647), or gelatin buffer (Sigma-Aldrich, G8327) for blocking membrane and for antibody solutions. Loading was controlled by developing membranes for GAPDH or by dying them with Ponceau S in all experiments. At least three independent measurements were carried out in each experiment. The following antibodies were applied: antiLC3B (SantaCruz, sc-16755), antiCaspase3 (SantaCruz, sc-7272), antiPARP (Cell Signaling, 9542S), antiULK-555-P (Cell Signaling, 5869S), antiULK (Cell Signaling, 8054S), antip70S6-P (Cell Signaling, 9234S), antip70S6 (SantaCruz, sc-9202), anti4-EBP1-P (Cell Signaling, 9459S), anti4-EBP1 (Cell Signaling, 9644S), antiGADD34 (SantaCruz, sc-8327), antieiF2α-P

(Cell Signaling, 9721S), antieiF2α (Cell Signaling, 9722S), antiAMPK-P (Cell Signaling, 2531S), antiAMPK (Cell Signaling, 2603S) and antiGAPDH (Santa Cruz, 6C5), and HRP-conjugated secondary antibodies (SantaCruz, sc-2354 and Cell Signaling, 7074S and 7076S). The bands were visualised using chemiluminescence detection kit (Thermo Scientific, 32106).

2.4. RNA Interference. RNA interference experiments were performed using Lipofectamine RNAi Max (Invitrogen) in GIBCO™ Opti-MEM I (GlutaMAX™-I) reduced-serum medium liquid (Invitrogen) and 20 pmol/ml siRNA. The siGADD34 oligonucleotides were purchased from ThermoFisher (HSS177543), and the siULK oligonucleotides were purchased from Ambion (AM16708). 200000 HEK293T cells were incubated at 37°C in a CO_2 incubator in antiobiotic-free medium for 16 hours, and then the RNAi duplex-Lipofectamine™ RNAiMAX (Invitrogen, 13778-075) complexes were added to the cells for overnight. Then fresh medium was added to the cells, and the appropriate treatment was carried out. To check the efficiency of GADD34 silencing, Western blot was used with GADD34 monoclonal antibody (SantaCruz, sc-373815).

2.5. RNA Extraction and Real-Time PCR. Total RNA content of cells was extracted using TRIzol RNA isolation reagent (Invitrogen) [33]. Retrotranscription was performed using SuperScriptII First-Strand Synthesis System (Invitrogen). Nucleic acid levels were measured using GenQuant pro RNA/DNA calculator. Equal amounts of cDNA were used for real-time PCR to check the efficiency of GADD34 silencing. PCR reaction and real-time detection were performed using GoTaq(R) qPCR Master Mix (Promega, A6002) and STRATAGENE Mx3005P Real-Time PCR Detection System. The real-time PCR thermocycles were the following: 95°C 10 min (1x), 95°C 30 sec, 58°C 45 sec, 72°C 30 sec (40x), 95°C 5 min, 55°C 1 min, and 97°C 30 sec (1x). The appropriate forward and reverse real-time PCR primers were used for GADD34 and GAPDH.

2.6. Cell Viability Assays. The relative amount of viable cells was calculated by Burker chambers. Cell viability was detected using CellTiter-Blue assay (Promega, G8080). Cells were grown and treated on 96-well plates and were incubated with resazurin for 2 h at 37°C. Absorbance was measured at 620 nm and expressed in arbitrary unit, being proportional to cell toxicity. At least three parallel measurements were carried out for each of these experiments.

2.7. Statistics. For densitometry analysis, Western blot data were acquired using ImageJ software. The relative band densities were shown and normalized to an appropriate total protein or GAPDH band used as reference protein (see Supplementary Information available here). For each of the experiments, three independent measurements were carried out. Results are presented as mean values ± S.D. and were compared using ANOVA with Tukey's multiple comparison post hoc test. Asterisks indicate statistically significant difference from the appropriate control: $^{*}p < 0.05$; $^{**}p < 0.01$.

3. Results

3.1. EGCG Affects Autophagy and Apoptosis in a Dose-Dependent Manner. The beneficial health effect of EGCG has been widely studied. It has been shown that low concentrations of EGCG enhance viability of HepG2 cells; however, its high concentration causes a significant decrease in the number of viable cells [34]. Experimental data have also revealed that EGCG is able to enhance both autophagy and apoptosis [35]. In order to figure out whether these cellular processes are correlated to cell viability during EGCG treatment, we further explored the role of green tea polyphenol in cellular decision-making process between life and death. First, human embryonic kidney cells (HEK293T) were treated with various concentrations (10, 20, 40, and 80 μM) of EGCG for 24 h, and we monitored both the relative number of viable cells and cell viability (Figure 1(a)). Corresponding to the already published data, we could confirm that low concentrations of EGCG (i.e., 10 and 20 μM) resulted in a slight increase in cell viability, while excessive level of the polyphenol (i.e., 80 μM) reduced the amount of viable cells by ≈50%.

To detect the activation profile or level of the key indicators of autophagy (such as LC3II and ULK-555-P) and apoptosis (procaspase-3, cleaved PARP) during EGCG treatment, immunoblotting was performed (Figures 1(b) and S1). At low concentration of EGCG (i.e., 10 and 20 μM), a high ratio of LC3II/LC3I and an intensive phosphorylation of ULK-555-P were observed indicating that cell viability is maintained in an autophagy-dependent manner by EGCG in a well-defined concentration range. However, a high concentration of EGCG (80 μM) slightly decreased the activity of autophagy, which was accompanied by a decrease in procaspase-3. Active capase-3 is able to cleave PARP; however, we did not observe any PARP cleavage suggesting that EGCG-dependent apoptosis might occur at a higher concentration of the polyphenol. Interestingly, ER stress was already observed at low concentration of EGCG (see eiF2α-P and GADD34 level in Figures 1(b) and S1), although the amount of phosphorylated eiF2α was reduced at an excessive level of this natural compound.

It is well-known that EGCG enhances AMPK, but its negative effect on mTOR has not been thoroughly studied yet. To investigate the role of EGCG in modifying the cellular balance of AMPK-mTOR pathways, we detected the key markers of AMPK (AMPK-P, ULK-555-P) and mTOR pathways (such as 4-EBP1-P and p70S6-P) by immunoblotting (Figures 1(b) and S1). EGCG treatment significantly enhances AMPK (see the phosphorylated status of both AMPK and ULK in Figures 1(b) and S1) while mTOR became inactivated. This was detected by both the dephosphorylation of p70S6 and the appearance of the lowest phosphorylation band of 4-EBP1 (Figures 1(b) and S1).

Taken together, these results further confirm that EGCG-induced autophagy is not hazardous for human cells but rather helps maintain cell viability; however, excessive level of this polyphenol might promote an apoptotic cell death. Low dose of EGCG is sufficient to activate AMPK and inhibit

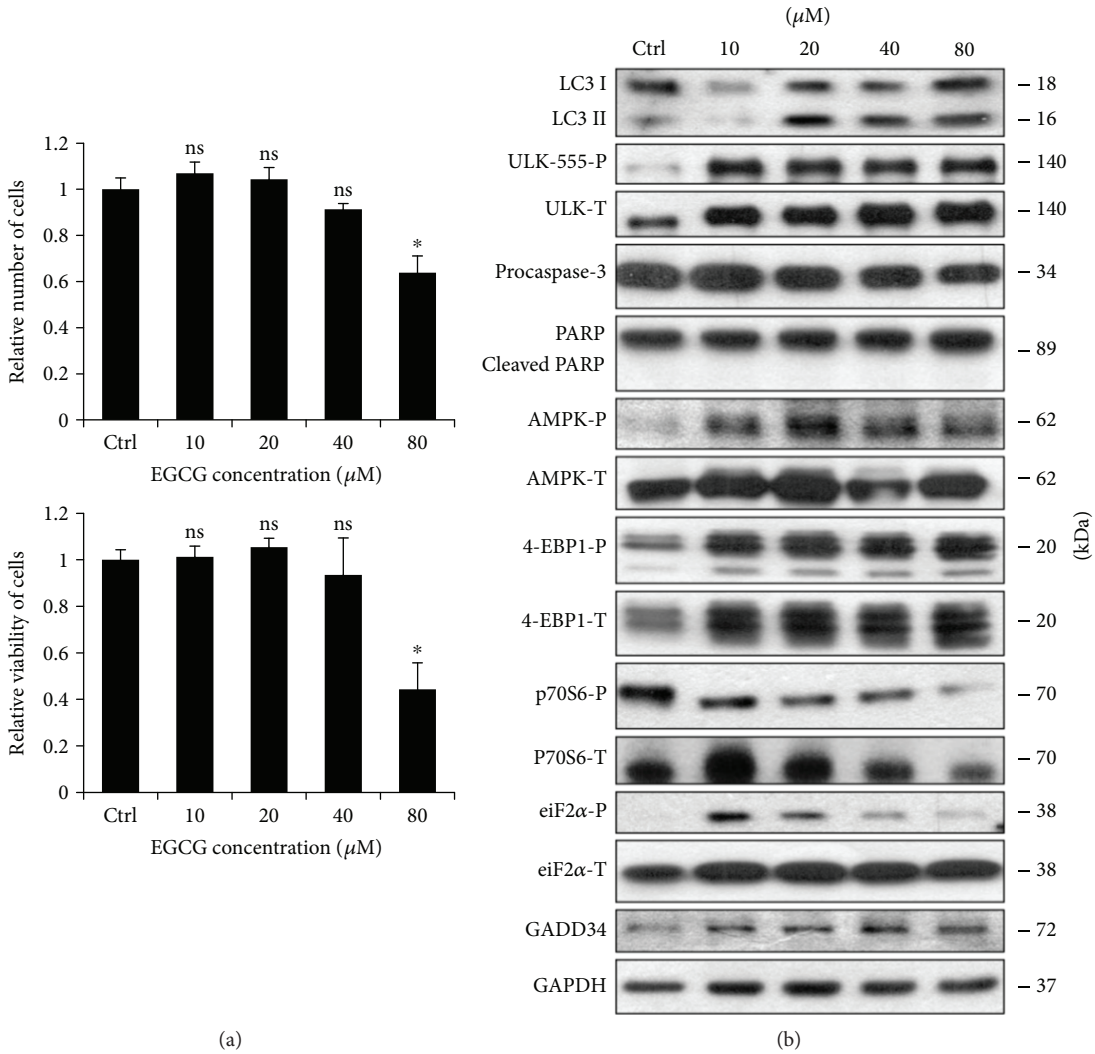

Figure 1: EGCG induces autophagy in a concentration-dependent manner. HEK293T cells were treated with 10, 20, 40, and 80 μM EGCG for 24 h. (a) Meanwhile, the relative number of viable cells (upper panel) and relative cell viability (lower panel) were denoted. (b) During EGCG treatment, the markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P, p70S6-P), as well as ER stress markers (i.e., eiF2α-P and GADD34) were followed by immunoblotting. GAPDH was used as loading control. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^*p < 0.05$.

mTOR suggesting that EGCG has a key role in unbalancing AMPK-mTOR pathways.

3.2. EGCG Induces Autophagy through mTOR-AMPK Pathways. To further explore that EGCG-induced autophagy via unbalancing AMPK-mTOR pathways, we used various drugs to enhance autophagy. It is well known that rapamycin (Rap) treatment induces autophagy via mTOR downregulation [19], while H-89 is a PKA inhibitor and promotes an mTOR-independent autophagy [36] (Figure S2). In order to understand EGCG-induced autophagy, we treated HEK293T cells with either Rap (100 nM, 2 h) or H-89 (2.5 μM, 2 h) and EGCG (20 μM, 24 h) without/with a subsequent Rap (100 nM, 2 h) or H-89 (2.5 μM, 2 h) addition.

We found that combined treatments (i.e., H-89 + EGCG and Rap + EGCG) did not cause a remarkable decrease in either cell viability or the relative amount of viable cells (Figure S3). Next, the key markers of autophagy, AMPK and mTOR pathways, were detected by immunoblotting (Figure 2). The Rap + EGCG treatment did not cause any additive effect on autophagy induction, AMPK activation, and mTOR downregulation, suggesting that both EGCG and Rap act via the same pathway to induce autophagy. Interestingly, the combined treatment with EGCG and H-89 had a significant additive effect on autophagy induction, indicating that EGCG and H-89 employ different pathways to promote autophagy. Although H-89 itself did not modify the balance of AMPK-mTOR pathways, EGCG was able to activate AMPK (see the phosphorylation of AMPK in Figure 2) and downregulate mTOR (see 4-EBP1-P in Figure 2) in the combined treatment.

Our combinatory treatment experiments suggest that EGCG does not activate autophagy in a PKA-dependent

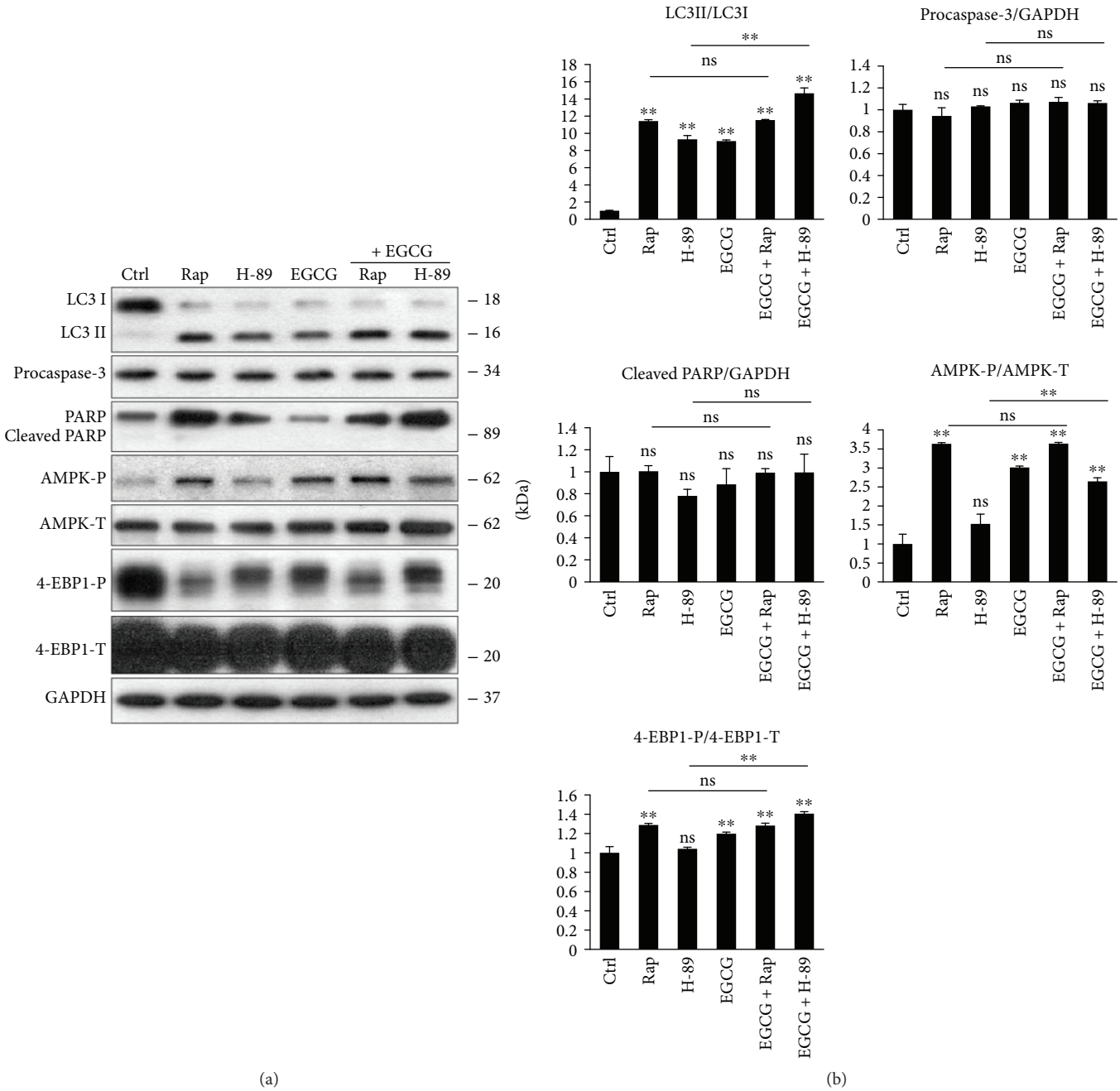

Figure 2: mTOR pathway is essential for EGCG-dependent autophagy induction. HEK293T cells were treated with rapamycin (Rap—100 nM, 2 h), H-89 (2.5 μM, 2 h), and EGCG (20 μM, 24 h) without/with followed by Rap (100 nM, 2 h) or H-89 (2.5 μM, 2 h) addition. (a) The markers of autophagy (LC3), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P) were followed by immunoblotting. GAPDH was used as loading control. (b) Densitometry data represent the intensity of procaspase-3, cleaved PARP normalized for GAPDH, LC3II normalized for LC3I, AMPK-P normalized for total level of AMPK, and 4-EBP1-P normalized for total level of 4-EBP1. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{**}p < 0.01$.

manner. Similarly to Rap, EGCG rather induces autophagy via unbalancing AMPK-mTOR pathways.

3.3. ULK1 Is Essential for the EGCG-Induced Autophagy. It is well-known that both AMPK and mTOR regulate autophagy through the phosphorylation of ULK1, one of the key control elements of this cellular process [21]. While AMPK stimulates ULK1 via phosphorylating its Ser-555 and Ser-777, mTOR inhibits autophagy by phosphorylation of different Ser residues in ULK1 (i.e., Ser-757) [21]. Therefore, to further confirm the role of AMPK-mTOR pathways in EGCG-induced autophagy, the effect of EGCG- (20 μM, 24 h)

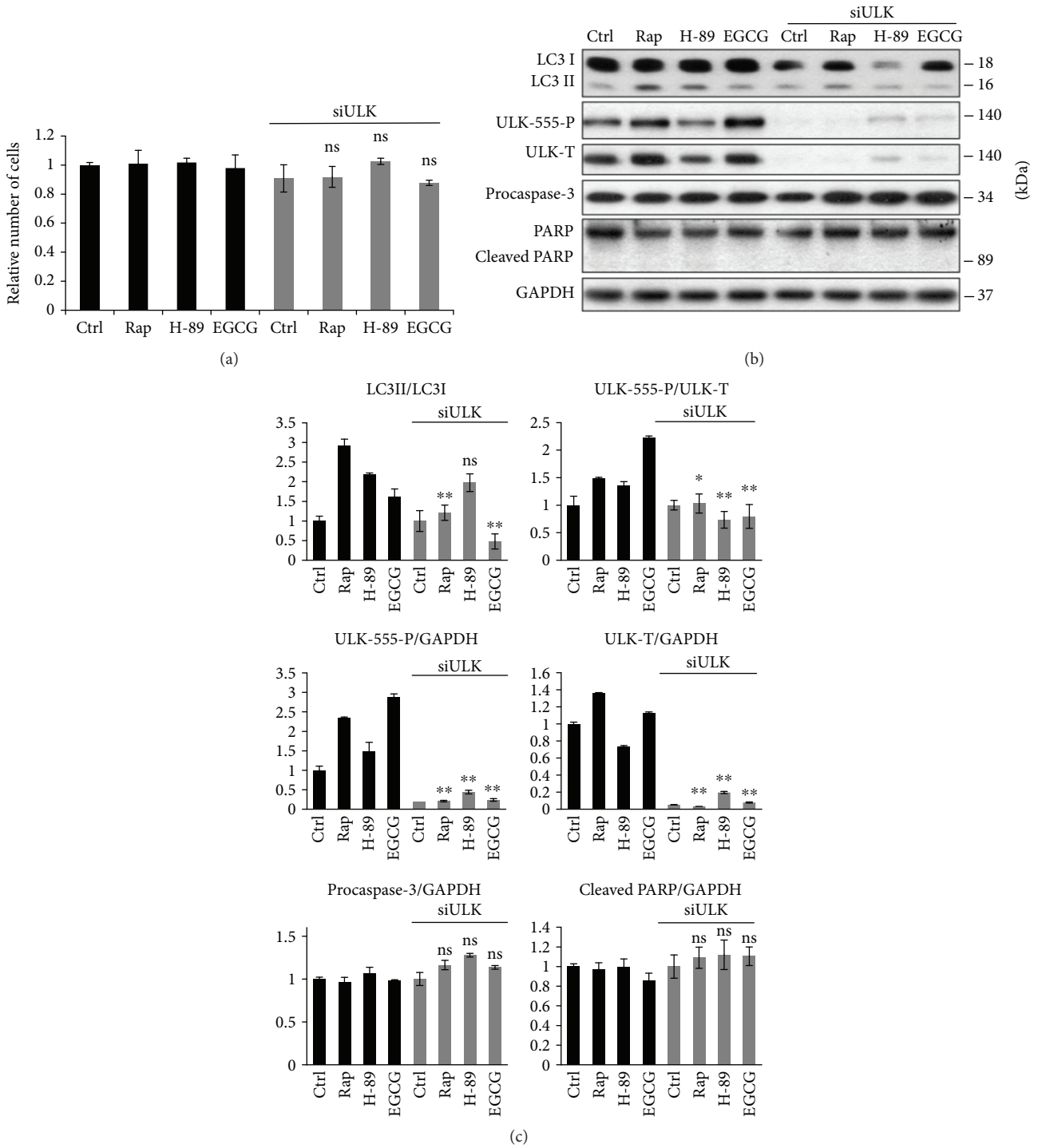

Figure 3: ULK1 is essential for EGCG-dependent autophagy induction. HEK293T cells were treated with rapamycin (Rap—100 nM, 2 h), H-89 (2.5 μM, 2 h), and EGCG (20 μM, 24 h) without/with followed by Rap (100 nM, 2 h) or H-89 (2.5 μM, 2 h) addition. (a) Meanwhile, the relative number of viable cells was denoted. (b) The markers of autophagy (LC3, ULK-555-P) and apoptosis (procaspase-3, PARP) were followed by immunoblotting. GAPDH was used as loading control. (c) Densitometry data represent the intensity of procaspase-3, cleaved PARP, and ULK-555-P and total level of ULK1 normalized for GAPDH, LC3II normalized for LC3I, and ULK-555-P normalized for total level of ULK. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

induced autophagy was detected in the presence or absence of ULK1 (Figure 3). We carried out Rap (100 nM, 2 h) and H-89 (2.5 μM, 2 h) treatments for controls. ULK1 knockdown using siULK did not affect the relative amount of viable cells suggesting that ULK depletion did not induce cell death (Figure 3(a)).

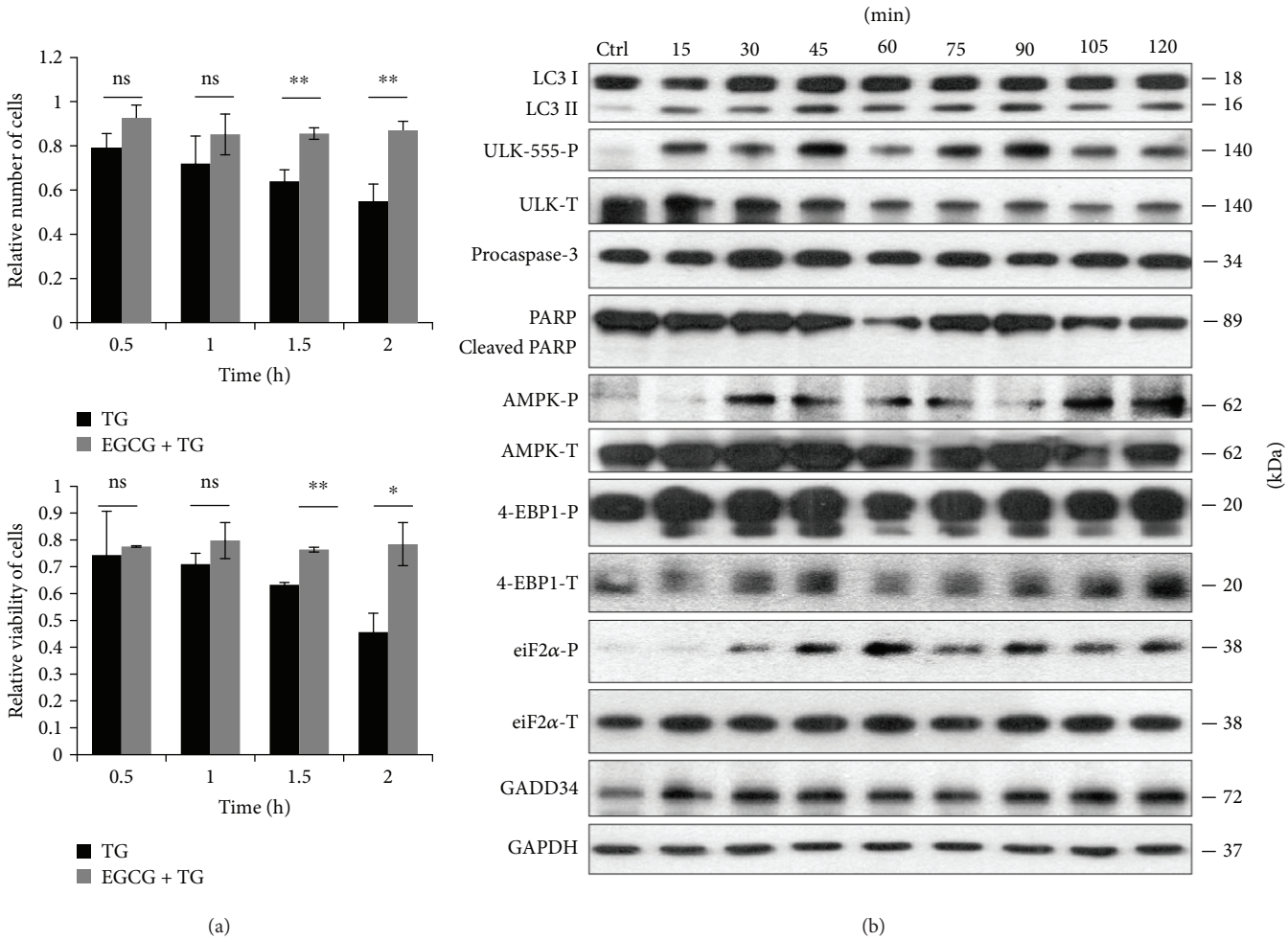

Figure 4: EGCG pretreatment extends autophagy-dependent survival with respect to TG-induced ER stress. HEK293T cells were pretreated with 20 μM EGCG for 24 h followed by TG (10 μM) treatment for 2 h. (a) Meanwhile, the relative number of viable cells (upper panel) and relative cell viability (lower panel) were denoted in time. (b) The markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P), as well as ER stress markers (i.e., eiF2α-P and GADD34) were followed by immunoblotting in time. GAPDH was used as loading control. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

Depletion of ULK1 abolished EGCG-induced autophagy (see the low LC3II/I ratio in Figures 3(b) and 3(c)). Similarly to EGCG, Rap was not able to promote autophagy in the absence of ULK1, while siULK did not affect the H-89-induced autophagy (see the LC3II/I ratios in Figures 3(b) and 3(c)). These results further confirm that AMPK-mTOR-regulated autophagy is independent from the PKA pathway.

Taken together, we could conclude that ULK1 is involved in EGCG-induced autophagy and in shifting the balance of mTOR-AMPK pathways.

3.4. EGCG Delays Apoptotic Cell Death at an Excessive Level of ER Stress. We have recently identified various drugs (such as metyrapone and resveratrol), which imbalance mTOR-AMPK pathways and thus induce autophagy-dependent survival in ER stress [31, 37]. Since EGCG affects the activation of AMPK and mTOR, we examined whether EGCG also has a positive effect on cell survival during ER stress.

In order to verify the role of EGCG in ER stress, HEK293T cells were pretreated with EGCG (20 μM, 24 h) and then an ER stressor was added, such as thapsigargin (10 μM, 2 h) or tunicamycin (25 μM, 2 h). While thapsigargin (TG) disrupts the calcium storage of the ER, tunicamycin (TM) inhibits N-linked glycosylation of secretory and membrane proteins in the ER [38, 39]. We have already shown that TM- or TG-induced ER stress occurs a transient peak of autophagy-dependent survival followed by apoptotic cell death [29]. To explore whether EGCG is capable to maintain cell viability upon ER stress, both the relative amount of viable cells and relative cell viability were detected during EGCG + TG or EGCG + TM treatments (Figures 4(a) and 5(a)). Addition of EGCG prior to TG or TM significantly extended cell viability and postponed cell death even at continuous treatments with an excessive level of the ER stressor. Our result suggests that this polyphenol is capable of improving cell viability.

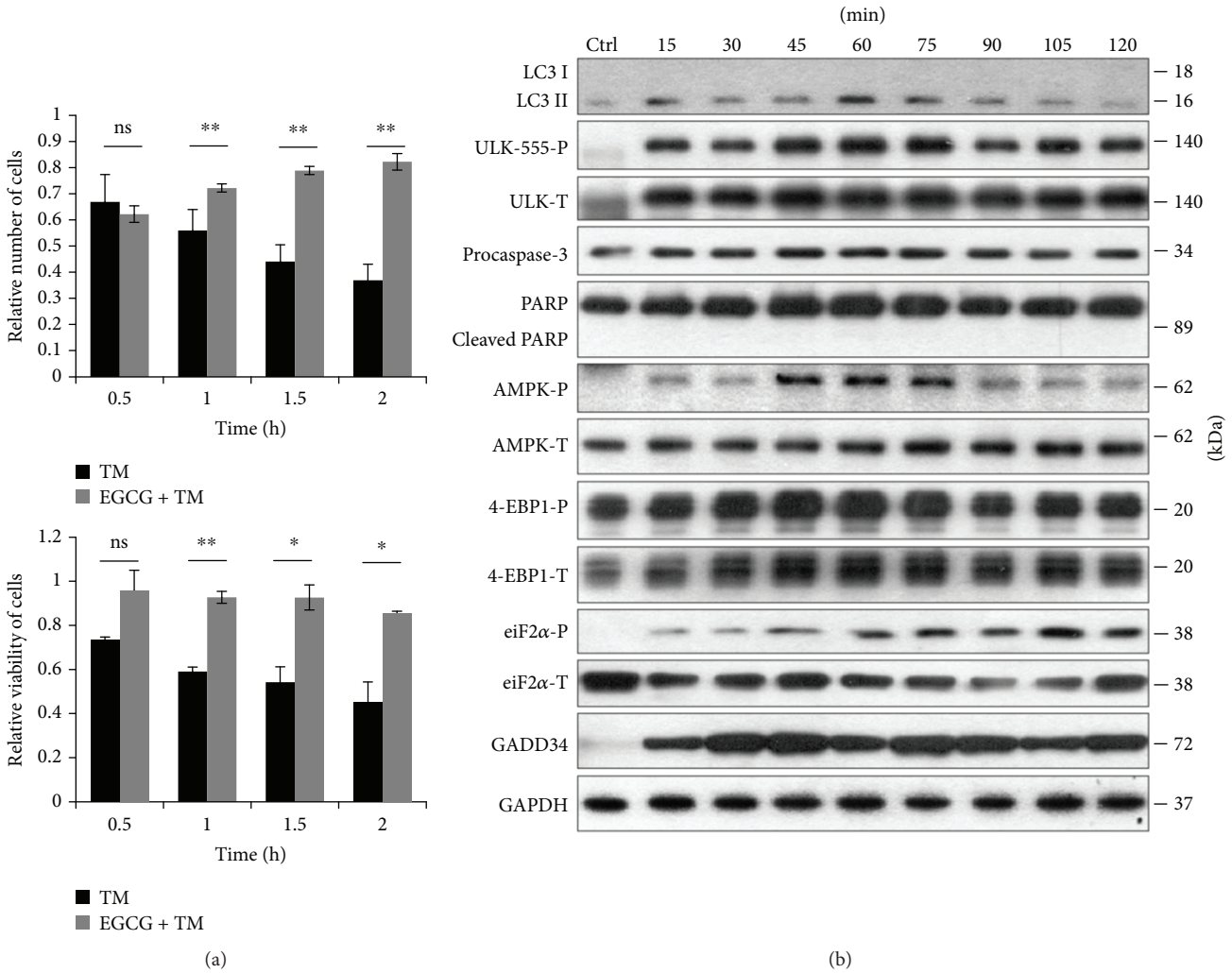

Figure 5: EGCG pretreatment extends autophagy-dependent survival with respect to TM-induced ER stress. HEK293T cells were treated with 20 μM EGCG for 24 h followed by TM (25 μM) treatment for 2 h. (a) Meanwhile, the relative number of viable cells (upper panel) and relative cell viability (lower panel) were denoted in time. (b) The markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P), as well as ER stress markers (i.e., eiF2α-P and GADD34) were followed by immunoblotting in time. GAPDH was used as loading control. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

In order to detect the effect of EGCG with respect to ER stress, autophagy, apoptosis, AMPK, and mTOR markers were followed during EGCG + TG or EGCG + TM treatments in time by immunoblotting (Figures 4(b), 5(b), S4, and S5). A remarkably high level of LC3II/I suggested that autophagy remained active even after a two-hour-long TG or TM treatment, while neither a drop in procaspase-3 nor the cleavage of PARP was observed. These results indicate that EGCG is able to postpone apoptotic cell death via autophagy induction upon an excessive level of ER stress.

The intensive phosphorylation of both AMPK and ULK (on its Ser-555 residue) suggests that AMPK got stimulated and remained active until the end of the combined treatment (Figures 4(b), 5(b), S4, and S5). The mTOR pathway got downregulated when ER stress was preceded with EGCG addition (see the 4-EBP1-P in Figures 4(b), 5(b), S4, and S5).

These results indicate that EGCG induces autophagy via unbalancing mTOR-AMPK pathways, and by this means it delays apoptotic cell death in ER stress.

3.5. Addition of EGCG Can Rescue GADD34 Inhibition with Respect to ER Stress. Recently, we have suggested that one of the key elements of ER stress response mechanism, called GADD34 (the growth arrest and DNA damage-inducible protein) associated with PP1 (protein phosphatase 1), constitutes a mechanistic link between ER stress and mTOR activation [37]. It has been also suggested that GADD34 promotes autophagy-dependent survival via downregulating mTOR in ER stress or in the stress caused by the expression of mutant huntingtin proteins [37, 40]. Inhibition of GADD34 by a PP1 inhibitor (i.e., guanabenz) or transfection with siGADD34 results in a downregulation

of autophagy-dependent survival and a quick activation of mTOR pathway, followed by apoptotic cell death during ER stress [37]. Both rapamycin and resveratrol treatments are able to diminish the negative effect of GADD34 downregulation by promoting autophagy induction, AMPK upregulation, and mTOR inhibition [37]. Since EGCG seems to be a potential regulator of mTOR-AMPK balance upon cellular stress, the polyphenol might protect the cells via autophagy induction even in the absence of GADD34 under ER stress.

GADD34 protein level got activated quickly when ER stress was preceded by EGCG addition (Figures 4(b), 5(b), S4, and S5). We observed that GADD34 level remained high even after 2 h long treatment with TM or TG supposing its important role in EGCG-induced autophagy with respect to ER stress.

To explore whether EGCG pretreatment can rescue GADD34 downregulation-induced apoptotic cell death upon ER stress, we carried out a combined treatment. First, HEK293T cells were treated with a GADD34 inhibitor, called guanabenz (GB—5 μM, 1 h), followed by EGCG addition (20 μM) for 24 h. Then ER stress was induced by TG (10 μM, 2 h) or TM (25 μM, 2 h). EGCG pretreatment was able to extend cell viability and increase the relative amount of viable cells in GB-pretreated cells under ER stress (Figure S6).

We analysed the effect of GADD34 inhibition during ER stress combined with/without EGCG addition via detecting autophagy, apoptosis, AMPK, and mTOR markers by immunoblotting (Figures 6 and 7). The inactivation of GADD34 was detected by eiF2α-P. In the absence of EGCG, GB quickly downregulates autophagy and induces apoptotic cell death during ER stress. However, when cells were pretreated with EGCG, the high ratio of LC3II/I indicates an intensive autophagy until the end of the treatment; meanwhile, apoptosis remains inactive. Neither procaspase-3 depletion nor PARP cleavage was detected in the presence of EGCG. Interestingly, EGCG was able to induce AMPK (see the intensive phosphorylation of both AMPK and ULK1 in Figures 6 and 7) and downregulate mTOR (see 4-EBP1-P in Figures 6 and 7) even if GADD34 was inhibited by GB during ER stress.

These data suggest that EGCG is able to maintain cell viability via autophagy-dependent survival even in the absence of GADD34 upon ER stress. Our experiments indicate that the negative effect of GADD34 inhibition by GB can be suppressed by EGCG-induced imbalance of mTOR-AMPK pathways with respect to ER stress.

3.6. GADD34 Silencing by siRNA Has Similar Effects to GB Treatment with Respect to ER Stress. To confirm that EGCG postpones ER stress-induced apoptotic cell death via GADD34, the combined treatment of ER stressor and EGCG was done in cells where GADD34 was silenced with siRNA (Figures 8 and S7). First, we tested the efficiency of siGADD34 both on mRNA (data not shown) and protein (Figure S7A) levels. Similar to addition of GB, GADD34 silencing drastically decreased the amount of viable cells during TG treatment, while pretreatment with 20 μM EGCG for 24 h was able to maintain cell viability (Figure S7B). Addition of TG in HEK293T cells expressing siGADD34 resulted in a short and dumped autophagic response (see the weak LC3II/I ratio and ULK1 phosphorylation in Figure 8), while an early apoptosis induction was observed, that is, depletion of procaspase-3 and appearance of cleaved PARP were already detected after 1.5 h long TG treatment. By contrast, EGCG pretreatment could maintain autophagy-dependent survival and delay apoptotic cell death even in the absence of GADD34 (Figure 8). Both LC3II/LC3I and ULK-555-P levels remain high; meanwhile, no caspase-3 activation was noticed. In these combined treatments, the AMPK also maintained its active state (see the constant phosphorylation of both AMPK and ULK1 in Figure 8), while the mTOR pathway remained blocked (see 4-EBP1-P in Figure 8). Similar effects were observed by using TM (data not shown).

These data further confirm that the negative effect of GADD34 silencing during ER stress can be rescued by EGCG addition. This natural compound is able to imbalance the AMPK-mTOR pathways and promote autophagy-dependent survival in the absence of GADD34.

4. Discussion

ER has a key function to maintain cellular homeostasis by containing some of the main regulatory elements of life-and-death decision. Consequently, ER stress-induced damages appear in lots of different human pathologies such as neurodegenerative diseases, obesity, type two diabetes, and many others [41–43]. Using both molecular and theoretical biological techniques, we have shown previously that apoptotic cell death is always preceded by autophagy-dependent survival upon excessive level of ER stress [29, 31]. Therefore, newly identified autophagy inducers might become potent drugs in the future by postponing the injurious effects of ER stress. We have recently confirmed that the "survival window" of autophagy can be expanded by pretreatment with mTOR inhibitors and/or AMPK activators (such as metyrapone and resveratrol) upon ER stress [31, 37]. Here, we introduce a new candidate for extending cell viability, namely, epigallocatechin-3-gallate (EGCG). Plant polyphenols, including green tea flavanols, have pleiotropic effects; however, many of their specific molecular targets have been recently identified. Flavanols are widely known as antioxidants, but under certain conditions (e.g., in the presence of ferric iron) behave as prooxidants [44]. Since they act mainly on cellular membranes, green tea flavanols are known to modulate various functions of the ER [45], including luminal enzyme activities [46, 47], membrane transport processes [48, 49], and redox homeostasis [47]. It has been also demonstrated that EGCG extends life expectancies significantly, which was attributed either to decreased oxidative stress and inflammation [10] or to the induced production of reactive oxygen species [50]. However, the involvement of the AMPK/SIRT1/FOXO axis seems to be firmly established.

Our data demonstrate that a low concentration of EGCG is able to induce autophagy (Figures 1(b) and S1) concomitantly with rise in cell viability, suggesting this

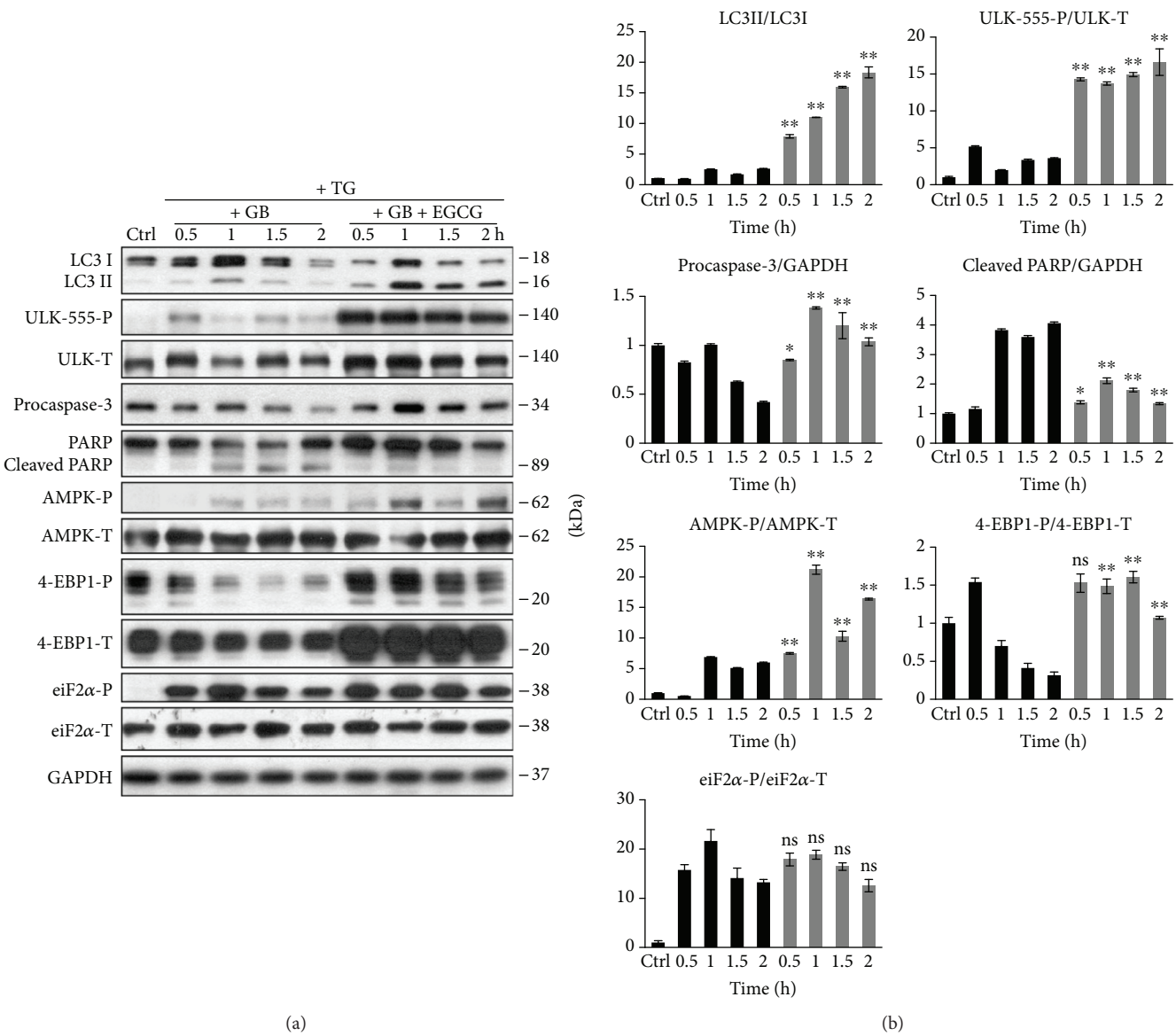

Figure 6: EGCG-dependent effect on mTOR-AMPK pathways rescues GADD34 inhibition with respect to TG-induced ER stress. HEK293T cells were pretreated with GB (5 μM, 1 h) then without/with EGCG (20 μM, 24 h) followed by TG addition (10 μM, 2 h). The GB level was kept high until the end of the cell treatment. (a) After TG treatment, the markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P), as well as ER stress markers (eiF2α-P) were followed by immunoblotting. GAPDH was used as loading control. (b) Densitometry data represent the intensity of procaspase-3, cleaved PARP normalized for GAPDH, LC3II normalized for LC3I, ULK-555-P normalized for total level of ULK1, AMPK-P normalized for total level of AMPK, 4-EBP1-P normalized for total level of 4-EBP1, and eiF2α-P normalized for total level of eiF2α. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

activation of self-eating process induced by the polyphenol is not harmful for the cells (Figure 1(a)). Pretreatment with low concentration of EGCG followed by addition of ER stressor (TG or TM) could extend autophagy-dependent survival (Figures 4(b), 5(b), S4, and S5); meanwhile, cell viability did not change (Figures 4(a) and 5(a)) and apoptosis (e.g., PARP cleavage) was not observed upon ER stress (Figures 4(b), 5(b), S4, and S5). Interestingly, Ahn et al. have indicated that the cytotoxic effect of excessive level of EGCG is due to the expression of ER stress response proteins, such as CHOP, GADD34, and ATF3 [34]. Here, we show that the translational initiation factor, eiF2α, gets phosphorylated even at low level of EGCG (Figures 1(b) and S1). Although eiF2α-P has a key role in shutting down the global protein translation upon ER stress, no cell death is observed suggesting that activation of ER stress response mechanism is not fatal. Rather this eiF2α phosphorylation induced by EGCG is essential to upregulate GADD34 level. In this study, we assume that GADD34 level is increased parallel to autophagy induction upon EGCG

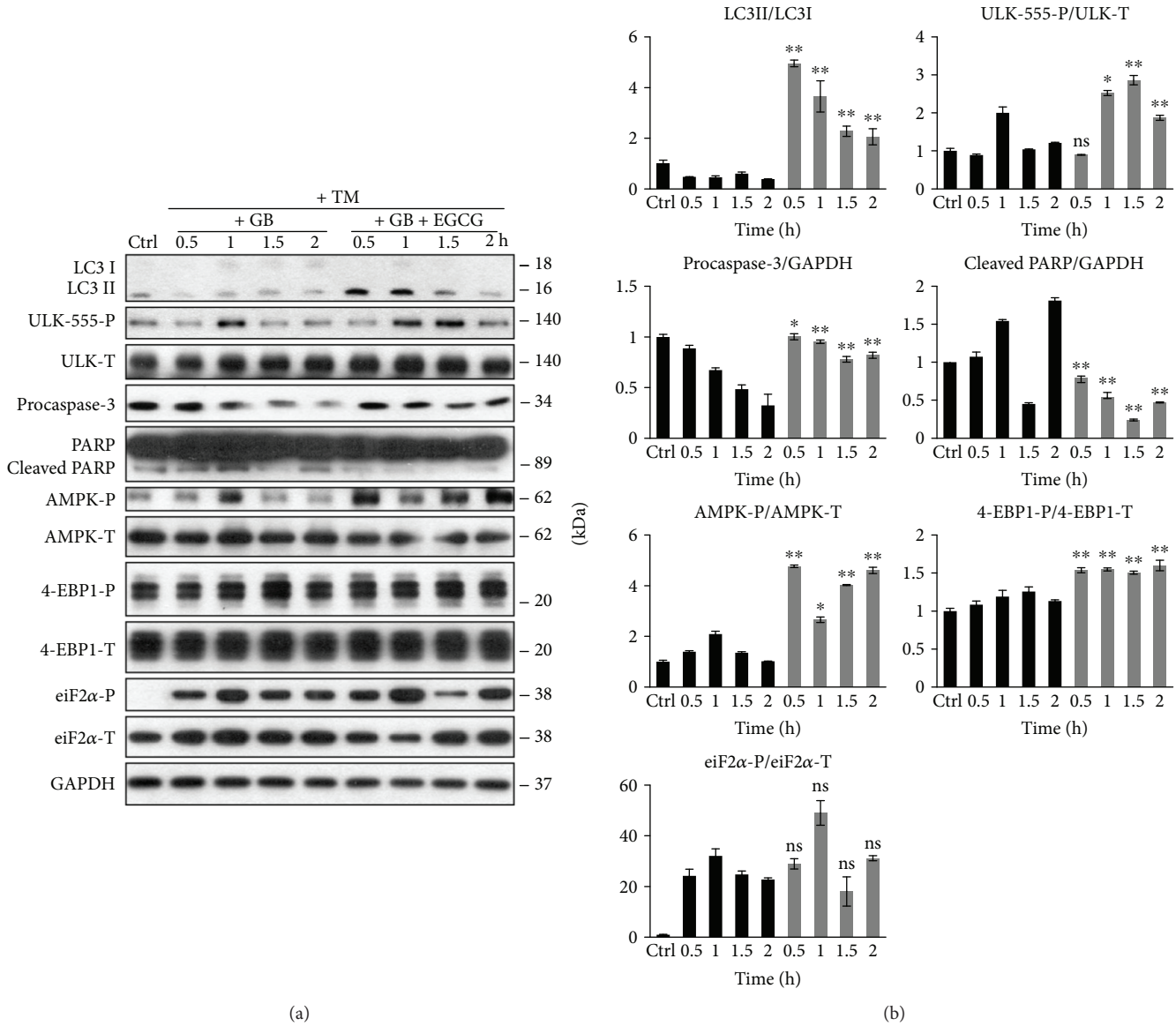

Figure 7: EGCG-dependent effect on mTOR-AMPK pathways rescues GADD34 inhibition with respect to TM-induced ER stress. HEK293T cells were pretreated with GB (5 μM, 1 h) then without/with EGCG (20 μM, 24 h) followed by TM addition (25 μM, 2 h). The GB level was kept high until the end of the cell treatment. (a) After TM treatment, the markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P), as well as ER stress markers (eiF2α-P) were followed by immunoblotting. GAPDH was used as loading control. (b) Densitometry data represent the intensity of procaspase-3, cleaved PARP normalized for GAPDH, LC3II normalized for LC3I, ULK-555-P normalized for total level of ULK1, AMPK-P normalized for total level of AMPK, 4-EBP1-P normalized for total level of 4-EBP1, and eiF2α-P normalized for total level of eiF2α. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

treatment (Figures 1(b) and S1), indicative of its important role in green tea polyphenol-induced cell survival.

Previously, we have shown that mTOR is downregulated with response to ER stress via GADD34 [37]. We have recently confirmed that blocking GADD34 results in a quick activation of both mTOR pathway and apoptotic cell death; meanwhile, AMPK gets downregulated and the period of autophagy-dependent survival is much shorter upon ER stress [37]. We also supposed that the negative effect of GADD34 depletion is successfully suppressed with mTOR inhibitors and/or AMPK activators (such as rapamycin and resveratrol) during ER stress [37]. To further confirm the role of EGCG in unbalancing mTOR-AMPK pathways, a pharmacological inhibitor (GB) or an siRNA was used to block GADD34 and then cells were pretreated with EGCG followed by addition of an ER stressor. In this study, we show that a 24-hour long pretreatment with a low concentration of green tea polyphenol followed by TG or TM addition was able to extend cell viability via intensive activation of both AMPK and autophagy; meanwhile, mTOR and

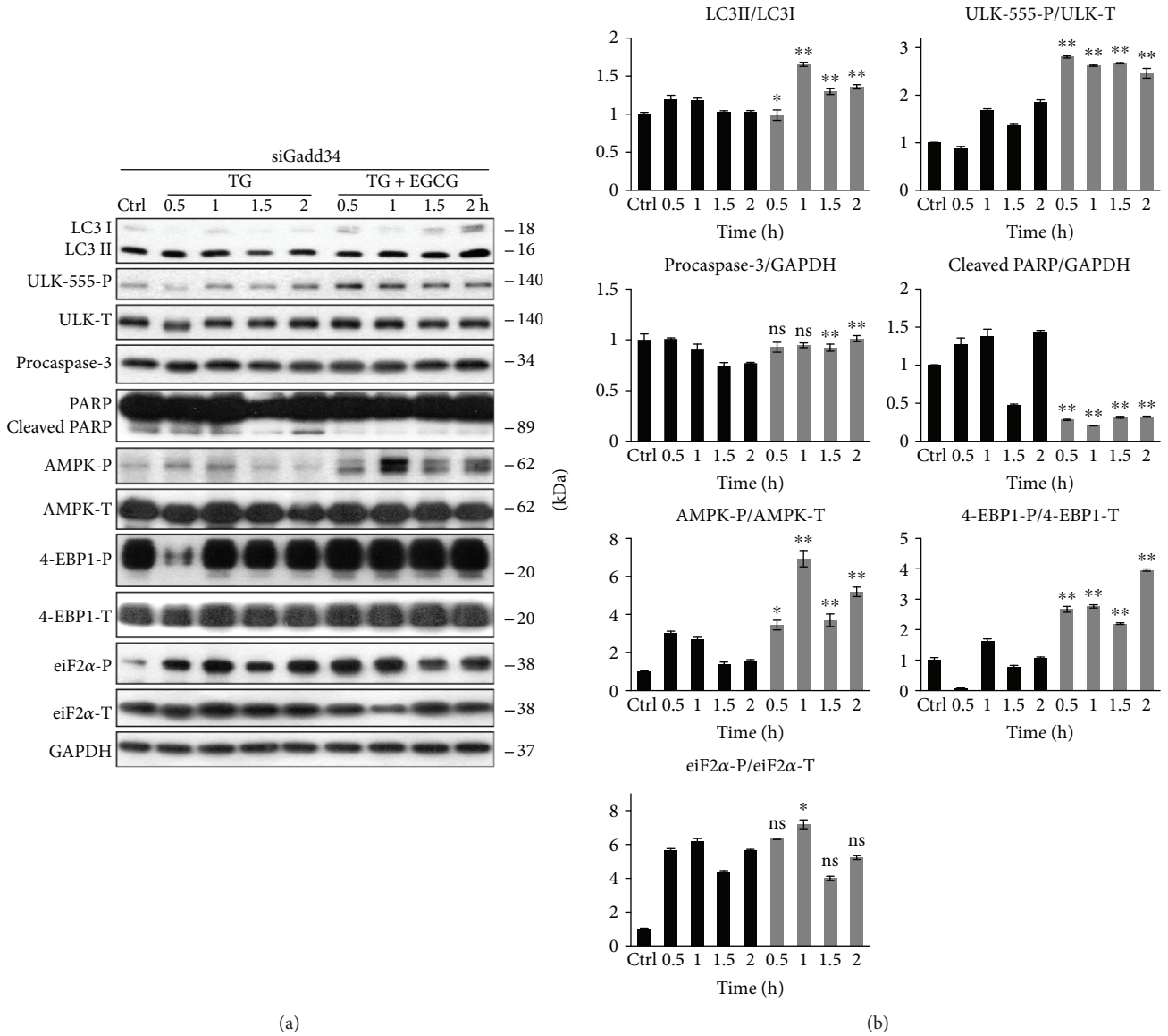

Figure 8: EGCG-dependent effect on mTOR-AMPK pathways rescues GADD34 depletion with respect to ER stress. GADD34 was silenced in HEK293T cells, and then cells were treated with 10 μM TG for 2 h or pretreated with EGCG (20 μM, 24 h) followed by TG addition (10 μM, 2 h). (a) After TG treatment, the markers of autophagy (LC3, ULK-555-P), apoptosis (procaspase-3, PARP), AMPK (AMPK-P), and mTOR (4-EBP1-P), as well as ER stress markers (eiF2α-P) were followed by immunoblotting. GAPDH was used as loading control. (b) Densitometry data represent the intensity of procaspase-3, cleaved PARP normalized for GAPDH, LC3II normalized for LC3I, ULK555-P normalized for total level of ULK1, AMPK-P normalized for total level of AMPK, 4-EBP1-P normalized for total level of 4-EBP1, and eiF2α-P normalized for total level of eiF2α. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, and asterisks indicate statistically significant difference from the control: $^{*}p < 0.05$; $^{**}p < 0.01$.

ER stressor-induced apoptotic cell death were downregulated (Figures 6, 7, and 8). Here, we suggest that EGCG treatment successfully modifies the balance of mTOR-AMPK pathways and thus the negative effect of GADD34 depletion was effectively suppressed. These results further confirm that EGCG-dependent fine-tuning of mTOR-APMK pathways has a crucial effect to maintain the precise balance of life-and-death decision under ER stress.

Since the effect of EGCG on mTOR pathway seems to be contradictory in the literature, EGCG treatment was combined with either mTOR-dependent (rapamycin) or PKA-dependent (H-89) autophagy promoter to identify which pathway is involved in autophagy induction in case of EGCG addition (Figure 2). Autophagy got similarly enhanced both in EGCG and EGCG + Rap treatments revealing that EGCG and Rap regulate the self-eating process via the same mTOR pathway. However, EGCG combined with H-89 significantly promoted autophagy compared to simple H-89 treatment (Figure 2), suggesting that EGCG-induced autophagy is not PKA-dependent. Similarly to rapamycin treatment, transfection with siULK drastically inhibited autophagy during EGCG treatment (Figure 3) confirming that green

tea polyphenol induces the self-eating process through ULK1-AMPK-mTOR regulatory network. Since AMPK downregulates mTOR pathway via direct phosphorylation, we cannot rule out that EGCG has both direct and indirect (through AMPK) negative effects on mTOR. Therefore, further studies are needed to identify the exact targets of EGCG.

In conclusion, the positive effects with pretreatment of precisely chosen concentration of EGCG in a human cell line are achieved via promoting autophagy-dependent survival. Therefore, green tea consumption or use of EGCG-loaded nanoparticles or capsules might have therapeutic role in the near future not only in the amelioration of the patients' symptoms suffering from ER stress-related diseases, and in the regulation of body weight as caloric restriction mimetic, but also—obviously not independently from the former effects—to expand lifespan of people. Our interesting findings highlight the potential of EGCG to extend life expectancy by unbalancing mTOR-AMPK pathways via GADD34 upon ER stress.

Abbreviations

EGCG: Epigallocatechin-3-gallate
mTOR: Mammalian target of rapamycin
AMPK: 5′ AMP-activated protein kinase
Rap: Rapamycin
GB: Guanabenz.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Supplementary Materials

Figure S1: EGCG induces autophagy in a concentration-dependent manner. Figure S2: the effect of H-89 treatment on cell viability. Figure S3: mTOR pathway is essential for EGCG-dependent autophagy induction. Figure S4: EGCG pretreatment extends autophagy-dependent survival with respect to TG-induced ER stress. Figure S5: EGCG pretreatment extends autophagy-dependent survival with respect to TM-induced ER stress. Figure S6: EGCG-dependent imbalance of mTOR/AMPK rescues GADD34 inhibition with respect to ER stress. Figure S7: EGCG-dependent imbalance of mTOR/AMPK rescues GADD34 depletion with respect to ER stress. *(Supplementary Materials)*

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Review Article

The Crosstalk between ROS and Autophagy in the Field of Transplantation Medicine

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Many factors during the transplantation process influence posttransplant graft function and survival, including donor type and age, graft preservation methods (cold storage, machine perfusion), and ischemia-reperfusion injury. Successively, they will lead to cellular and molecular alterations that determine cell and ultimately organ fate. Oxidative stress and autophagy are implicated in posttransplant outcome since they are both affected by the stress responses triggered in each step (donor, preservation, and recipient) of the transplantation process. Furthermore, oxidative stress influences autophagy and vice versa. Interestingly, both processes have positive as well as negative effects on graft outcome, suggesting they are tightly linked during the transplantation process. In this review, we discuss the importance, regulation and crosstalk of oxidative signals, and autophagy in the field of transplantation medicine.

1. Introduction

For patients with end-stage organ disease, organ transplantation has become the treatment of choice. However, the success of transplantation is limited by a global shortage of suitable organs as well as loss of grafts following transplantation due to primary nonfunction or rejection. The gap between supply and demand has steadily increased over the years and, as a result, so has the number of patients on the waiting list [1, 2]. This is an alarming increase, as organ transplantation significantly improves a patient's quality of life as well as survival rate when compared to patients who remain on the waiting list [3–5]. These problems can be addressed by increasing the use of older and higher risk donors, while simultaneously improving graft longevity.

Oxidative stress levels correlate with graft survival in all steps of the transplantation process including in the donor [6–16], during preservation [17, 18], and reperfusion in the recipient. In donation after brain death (DBD) donors, brain death pathophysiology leads to increased renal oxidative damage markers which correlate with acute rejection, delayed graft function (DGF), and allograft function [6–16]. In donation after cardiac death (DCD) donors, cardiac arrest causes warm ischemia which is associated with impaired graft function and higher mortality rates [6–8]. Increased oxidative stress markers are evident in organs from DCD donors, but no evidence is available correlating them with the outcome. Clinically proven donor treatments that benefit graft survival include dopamine administration and hypothermic cooling of DBD donors, of which the effects could be related to modulation of oxidative stress [19, 20].

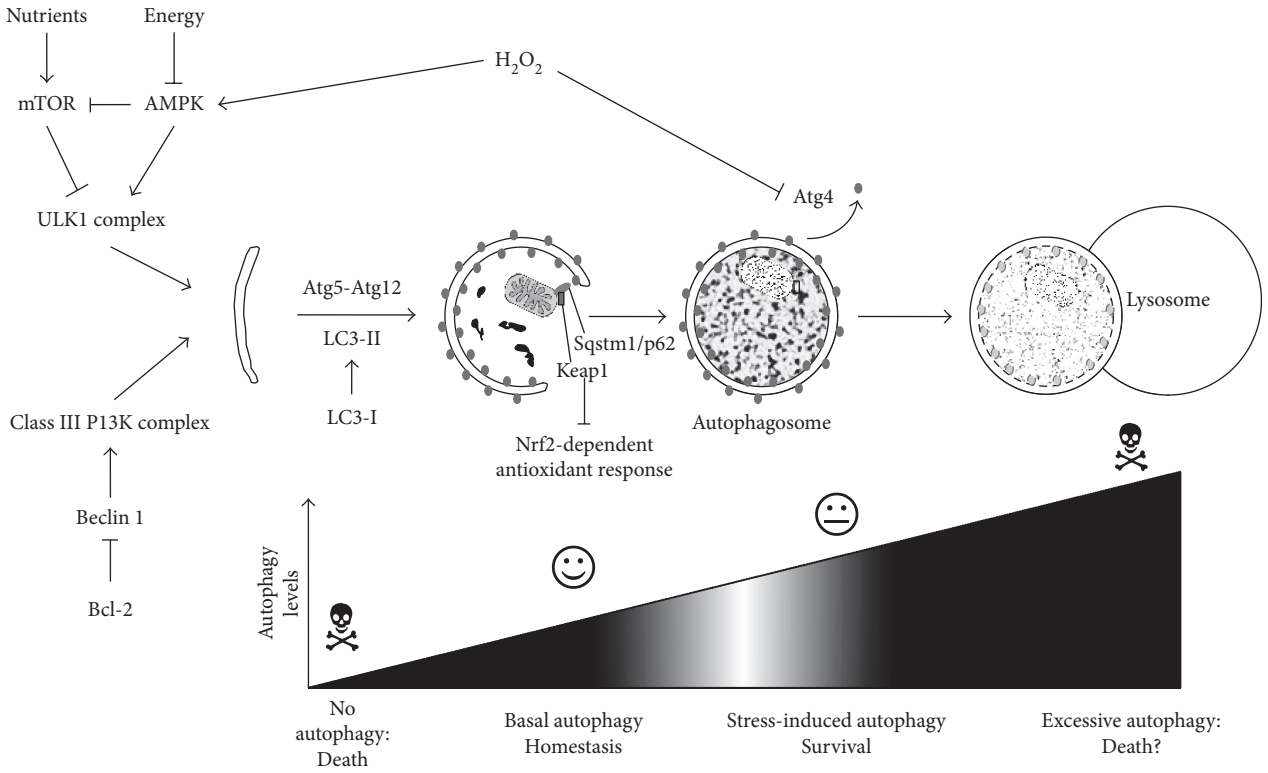

Figure 1: Overview of the autophagy process. Autophagy is initiated by the ULK1 complex, which is negatively regulated by mTOR, but positively by AMPK. This way it responds to nutrient or energy deprivation. In addition, class III PI3K complex requires Beclin 1, which is inhibited by Bcl-2. During elongation, Atg5-Atg12 and LC3-II are required. The latter is attached to the autophagosomal membranes. LC3-II will be delipidated on the outer membrane by Atg4 (a process inhibited by H_2O_2) but remains on the inner membrane and will be degraded inside the lysosomes. Mitochondria can be also degraded (mitophagy), via recruitment of Sqstm1/p62. The latter protein also recruits Keap1 for degradation, thereby enabling Nrf-2-dependent antioxidant transcription. Eventually, autophagosomes fuse with lysosomes, the cellular structures in which degradation takes place. The levels of autophagy determine the outcome on cellular injury and need to stay balanced in order not to provoke death.

Organ grafts suffer additional ischemic injury during preservation. Prolonged duration of cold ischemia is considered an independent risk factor for a nonfunctioning or dysfunctioning transplant, particularly in marginal or extended criteria donation (ECD) donors [21]. These side effects have recently led to the implementation of hypothermic machine perfusion (HMP). HMP has clear benefits over static cold storage, as evidenced by improved graft function and survival rates in kidney transplantation [22, 23], as well as reduced oxidative stress markers in experimental [24, 25] and clinical [26] liver preservation.

In the recipient, the mechanism of reperfusion injury, labeled ischemia-reperfusion (IR) injury (IRI), has been reported in most solid transplantable organs [27–31] and is mediated by reactive oxidative species (ROS) production most likely from donor-derived vascular cells [32, 33], followed by a second burst of ROS probably produced by the recipient's phagocytes [29, 34–40]. However, mitochondria are also implicated in ROS [29, 41–44] as well as nitric oxide (NO) production during IRI [45–47]. Despite overwhelming experimental evidence on the beneficial effects of attenuating ROS during this phase, clinical evidence remains limited.

Oxidative stress is a known inducer of autophagy. Even though autophagy regulation during the transplantation process is only starting to be understood, several autophagy modulators have already been implemented. In this review, we will first introduce autophagy in the context of the transplantation process and cover the current knowledge during each of these stages. Secondly, we will touch upon the complex, intertwined, and reciprocal relationship of oxidative stress and autophagy in the field of transplantation medicine while covering the therapeutic strategies that target each of these pathways.

2. Autophagy and Transplantation

2.1. Autophagy: Importance and Mechanisms. Several pathways of autophagy exist [48], of which macroautophagy is the best studied (and which will simply be referred to as "autophagy" for the remainder of this manuscript). It involves the formation of double-membranous vesicular "autophagosomes" that occlude and transport the soon-to-be degraded material to the lysosomes (Figure 1). This process is normally constitutively active in cells, albeit at a low basal level, thereby maintaining cellular homeostasis

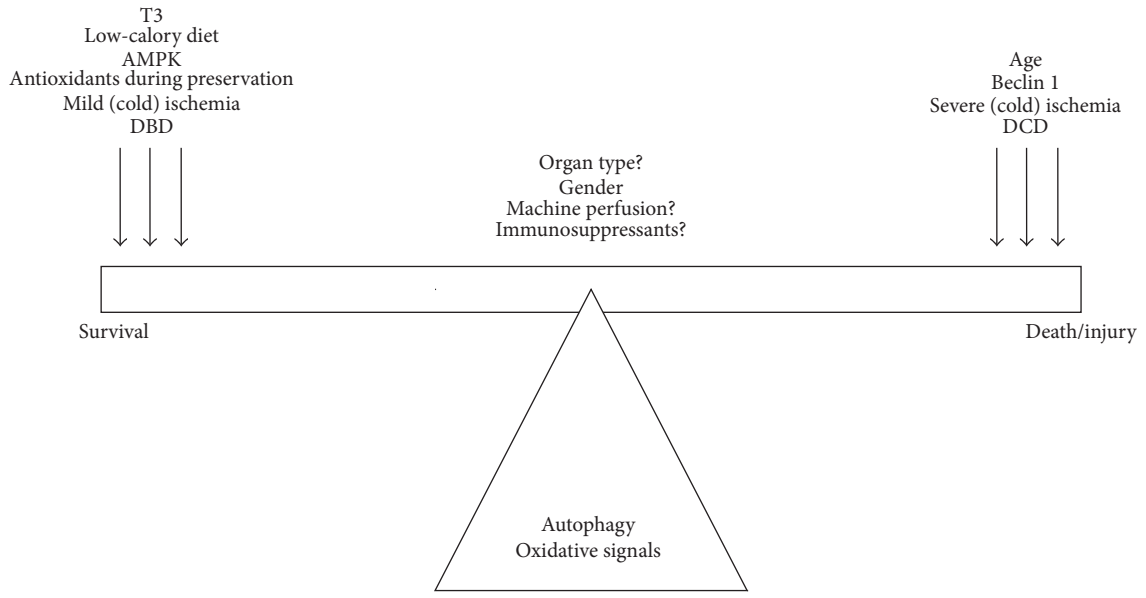

Figure 2: Regulation of oxidative signals and autophagy by transplantation-related factors. Excessive autophagy and/or oxidative stress can lead to increased graft injury and tilt the balance to the right. To tilt the balance towards survival, excessive signals need to be reduced towards protective levels of oxidative signals and autophagy.

(Figure 1). However, autophagy is stimulated upon stress through several signaling pathways, of which the mammalian target of rapamycin (mTOR) pathway is the most notable in response to nutrient stress. Energy deprivation attenuates mTOR signaling through the activation of AMP-activated kinase (AMPK), which also directly stimulates autophagy by the phosphorylation of the autophagy-initiating ULK1 complex. Another initiation complex is constituted by Vps34 (class III PI3K) and Beclin 1, an autophagy-specific BH3-only domain-containing protein that is inhibited by several antiapoptotic Bcl-2 family proteins (Figure 1). As such, apoptotic signals can also trigger autophagy, in which the autophagic response often precedes apoptosis as the first attempt to survival [49]. However, when autophagy fails, cells will eventually activate apoptosis, which may even occur with the help of the still active autophagic machinery. This shows that autophagy may switch from a prosurvival to a prodeath pathway under certain conditions [50], although the exact mechanism, context, and details on this autophagy-dependent cell death remain currently elusive.

Following initiation, the formation of autophagosomes is mediated by the Atg5-Atg12 complex and the formation of phosphatidylethanolamine-conjugated LC3 ("LC3-PE" or "LC3-II") (Figure 1). The latter is generally used as a marker for autophagy, as it is distinguishable from its precursor LC3-I on Western blot and its fluorescent labeling allows visualization of autophagosomes as GFP-LC3 punctae. Despite delipidation of LC3-II on the outer membrane by Atg4, LC3-II remains attached to the inner autophagosomal membranes even after fusion of autophagosomes with lysosomes. Therefore, an increase of LC3-II or GFP-LC3 punctae can signify stimulation of autophagy or attenuation of the final steps in autophagy (e.g., inhibition of fusion), leading to an accumulation of autophagosomes without any true upregulation of autophagy. Therefore, prudency is advised when interpreting autophagy data because the dynamic character of the "autophagic flux" should be taken into consideration [51].

As the graft endures several types of stress during transplantation, it is evident that the protective properties of autophagy might be important in restoring cellular homeostasis and function in the organ grafts. Interestingly, DGF increases with donor (and recipient) age as aging leads to increased susceptibility towards cellular stress [52, 53]. An underlying mechanism for this increased vulnerability of aged organs is the age-related reduction in autophagy [52]. It has therefore been suggested that pharmacological stimulation of autophagy could reduce graft injury and promote function [54]. However, as excessive autophagy may detrimentally impact cellular fate through autophagy-dependent cell death (Figure 1), it is important to first understand the dynamics and role of autophagy to determine whether autophagy stimulation or inhibition is the best option in transplantation.

2.2. Autophagy in the Donor. The autophagic response in the donor is likely the result of donor-related characteristics including donor age, gender, comorbidities, and donor type of death. Of course, ischemic time is strongly prolonged in DCD compared to DBD grafts, which could partially explain the stronger injury in DCD grafts. This is important, since autophagy's dynamics and role (protective versus detrimental) during ischemic stress are likely dependent on the extent of ischemic injury, at least in the kidney [55, 56] and heart [57]. As DCD donors suffer more extensive anoxic injury compared to DBD donors, this could trigger autophagy-dependent cell death (Figure 2) and suggests that therapeutic strategies involving autophagy modulation are strongly donor type-dependent.

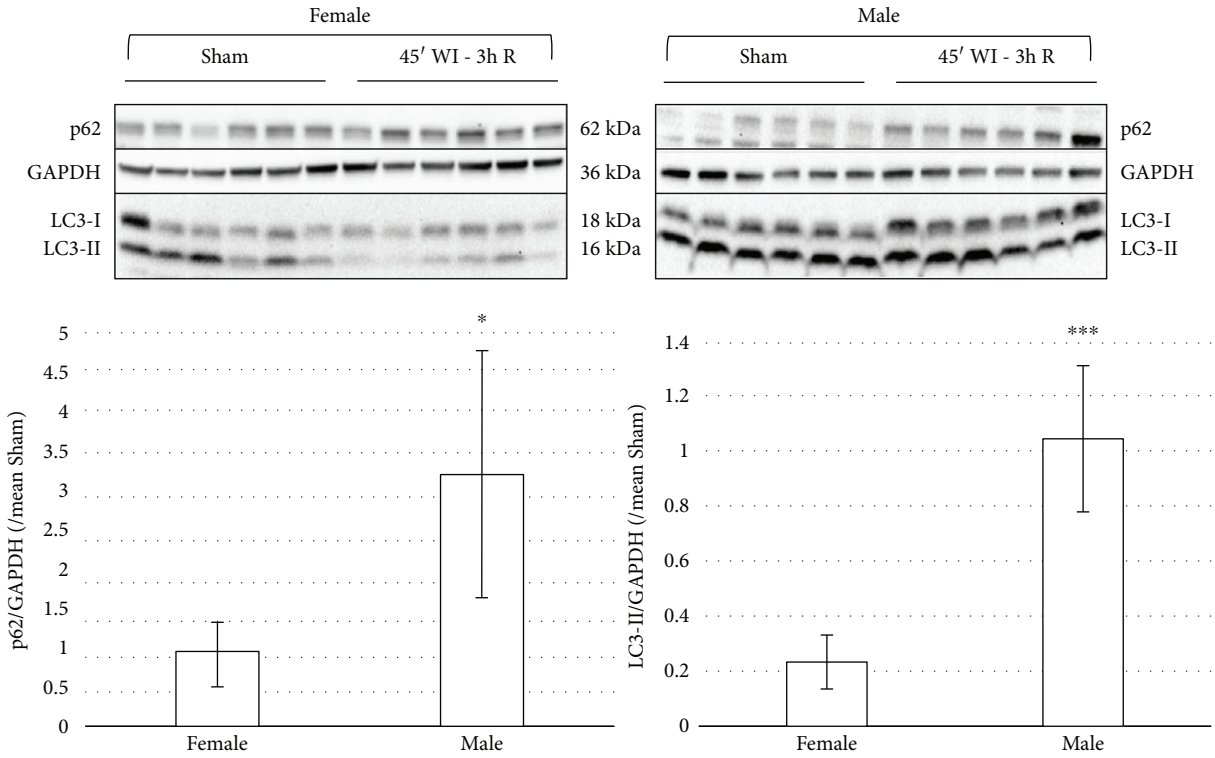

Figure 3: Gender differences in autophagy activation in response to ischemia-reperfusion injury. Western blot expression of autophagy-related proteins LC3-I, LC3-II, and Sqstm1/p62 in female and male Sprague-Dawley rat kidneys subjected to 45 min of warm ischemia (WI) followed by 3 h of reperfusion (3 h R). Each lane represents an independent experiment ($N = 6$). Quantification of p62 or LC3-II over GAPDH levels, compared to the mean of the corresponding Sham group. LC3-II and p62 are clearly lower in the WI group of females compared to the male WI group. Results are presented as mean ± SD ($N = 6$ per group) ($^{*}p < 0.05$; $^{***}p < 0.001$).

Additionally, the higher posttransplant injury in ECD (e.g., older) kidneys may be attributed to a decline in autophagic activity with age [58–61], justifying autophagy stimulation as the preferred strategy in these donors. Compared to young mice, old mice showed a decreased autophagic response in terms of vacuole formation and elimination after stimulation of these processes with vinblastine and Triton X-100 [60]. In line with this, hypoxia-induced injury was reduced by starvation-induced autophagy in older kidneys [62]. Besides donor age, a less studied but seemingly equally important feature is the gender of the donor. In a cardiac IRI study in mice, males and females showed different autophagic activities: male mice show a decrease, and female mice an increase in LC3-II levels [61]. We have also observed gender differences in the kidney, with a decrease in male, but unchanged LC3-II levels in female Sprague-Dawley rats subjected to 45 min of ischemia followed by 3 h of reperfusion (Figure 3). As such, therapeutic strategies involving autophagy modulation may differ in organs coming from male and female donors (Figure 2).

2.3. Autophagy during Organ Preservation. Mixed reports exist on the effects of cold ischemia on autophagy. In mouse kidneys, cold ischemia resulted in increased autophagy markers [63]. Interestingly, repression of autophagic flux by means of lysosomal inhibitor bafilomycin A1 resulted in less apoptosis, suggesting that autophagy could trigger cell death during renal cold ischemia. Similar findings were observed in cold-preserved rat lungs [61], where prolonged preservation resulted in increased autophagy associated with cell death. Alternatively, decreased markers of autophagy were found following cold ischemia in marginal, steatotic rat livers [64–66]. Induction of autophagy in these marginal livers by means of melatonin and trimetazidine addition to the cold preservation medium improved organ quality as evidenced by lower levels of injury markers ALT and GLDH [66]. These benefits were attenuated when autophagy was suppressed with bafilomycin A1 [66]. Furthermore, oxygen insufflation of the cold preservation medium of marginal livers reversed the suppression of autophagy and functional impairment [65], suggesting beneficial effects of autophagy induction. These studies suggest that the differential effects of autophagy activation might be organ-dependent (Figure 2; see also Autophagy in the Recipient) and closely associated with organ function and cell death.

2.4. Autophagy in the Recipient. The role of autophagy in IRI is best studied in the context of the transplantation process. However, its dynamics and roles remain elusive and are likely dependent on different factors. Firstly, when studying the nature of autophagy dynamics, that is, whether autophagy is stimulated or attenuated during IRI, research suggests that autophagy is mostly upregulated in the heart [67] and the kidney [55], while findings in the liver are

conflicting [64]. These discrepancies might be explained by several reasons (described in more detail in [55]), including the difficulty of measuring the dynamic process of autophagic flux in static samples. Indeed, an increase in LC3-II could equally well indicate stimulated autophagy as well as inhibited autophagic flux. *In vivo* experiments with chloroquine (an inhibitor of autophagosome-lysosome fusion, mimicking inhibited autophagic flux) suggest that autophagy inhibition might occur in the heart [68] and liver [69]. Moreover, as autophagy often fluctuates during prolonged stress, multiple time point postreperfusion should be investigated [55].

Secondly, conflicts arise when looking at the proposed role of autophagy during IRI. In hepatic IRI models, most studies indicate a protective role for autophagy [64], whereas both protective and detrimental roles are assigned to autophagy in the heart and kidney [55, 67]. Besides the difficulties in measuring autophagic flux, nonspecific chemical modulators of autophagy also have secondary effects on mTOR (e.g., rapamycin), PI3K (e.g., 3-methyladenine), or lysosomal and endocytic function (bafilomycin A1 and chloroquine) [55]. Even data in conditional autophagy knockout mice (e.g., $atg5^{-/-}$) are important to interpret with caution. As autophagy is an important mechanism for basal cellular homeostasis in cells (Figure 1), any stress addition in these models will likely lead to more injury than in wild-type. In this context, proximal tubule-specific Atg5 KO mice displayed strange concentric membranous structures in targeted cells [70], suggesting unhealthy cells.

Thirdly, several reports propose a dual role for autophagy in IRI, dependent of the extent of the stress [55, 71]; that is, mild IR stress leads to protective autophagy stimulation, while severe stress could trigger a switch towards autophagy-dependent cell death (Figures 1 and 2). In this respect, the duration of ischemia prior to transplantation is an important factor to consider. The longer the ischemic period, the more severe the reperfusion injury, which seems associated mostly with a detrimental role for autophagy, at least in the kidneys [55]. Besides the extent of the stress, the type of stress that initiates autophagy is also important. This is evident in the heart, where autophagy-dependent cell death during reperfusion seems to be dependent on the levels of Beclin 1, in which initiation is likely determined by apoptotic factors. Protective autophagy on the other hand seems to be dependent on AMPK activation and would therefore be related to changes in energy status (Figure 2) [71].

Finally, autophagy during IRI is strongly determined by the degree of autophagy dependency of the organs and even of different cell types within an organ (Figure 2). In renal podocytes, for example, autophagy is much more important for cellular homeostasis (as these cells are postmitotic) than in tubular cells. Also, cardiomyocytes are strongly dependent on basal autophagy, while hepatocytes rely more on stress-induced autophagy. In this respect, liver cells might have different mechanisms controlling autophagy as they can tolerate more severe stress than other organs. This might explain the more consistent data regarding the protective role for autophagy in this organ compared to the kidney and the heart during IR.

3. Autophagy and Oxidative Stress in Transplantation

3.1. The Relationship between Autophagy and Oxidative Stress. Both oxidative stress and autophagy have been described as both protective and detrimental pathways in response to cellular stressors [34, 55]. Therefore, it is feasible that the fine balance of oxidative stress levels and autophagy activation plays an important role in the long-term function and survival of organ grafts. This makes modulation of these pathways interesting targets to predict or improve graft function and survival after transplantation.

Surprisingly, the only currently known direct redox-based regulation of autophagy is the inhibitory oxidation of Atg4 by H_2O_2, which suppresses the delipidation of LC3-II [72] (Figure 1). Furthermore, H_2O_2 is also proposed to stimulate autophagy initiation directly via regulation of AMPK [73] (Figure 1). In a slower, more indirect fashion, oxidative stress also regulates transcription of Beclin 1 and LC3 [54]. Together, these protective, proautophagic effects are achieved with subtle changes in ROS. Conversely, acute and persistent ROS production can oxidatively modify macromolecules in such a way that they are only partially degraded by the autophagic/lysosomal pathway. This produces an indestructible product known as lipofuscin, which accumulates within the lysosomes, hampers their function, and sustains or even exacerbates oxidative injury [74]. Together, this suggests that the amount of ROS produced will determine whether autophagy will be activated or inhibited and whether it prevents or amplifies further damage.

Besides the regulation of autophagy by oxidative molecules, autophagy reciprocally regulates oxidative signals. As it is a clearance mechanism, autophagy may remove oxidatively damaged macromolecules or even entire organelles. Upon excessive ROS production, mitochondria risk severe damage and need to be partially removed [75]. This occurs through the selective autophagic degradation of damaged mitochondrial fragments called "mitophagy" (Figure 1). This process involves the recruitment of PINK1 and Parkin to the outer mitochondrial membrane where they promote ubiquitination (Ub) of several mitochondrial proteins [76, 77]. This Ub signal serves as a recognition for Sqstm1/p62, which is an adapter protein for LC3-II (Figure 1). The link between ROS and mitophagy was confirmed in a study on heart failure in mice, describing that p53-induced impairment of mitophagy resulted in mitochondrial dysfunction and increased ROS production [76, 77].

In addition to mitophagy, autophagy is shown to regulate antioxidant responses through interactions of Sqstm1/p62 with Keap1. Sqstm1/p62 recruits Keap1 via interactions with ubiquitinated aggregates, after which Keap1 is degraded via autophagy. Degradation of Keap1 in turn prevents it from ubiquitinating and degrading Nrf2-dependent transcription factors, thereby enabling the antioxidant response [78] (Figure 1).

3.2. Autophagy and Oxidative Stress in the Donor. The accumulation of acute and chronic injuries in the donor leads to increased senescence and reduced organ quality. In ECD

donors, this may be due to an increase in age-related oxidative damage (Figure 2) [16] as well as a reduction of autophagic activity and hence the inability to remove oxidatively damaged organelles. This link was shown in the kidneys of aged rats, where a high caloric diet exacerbated oxidative damage and aging, while autophagy was reduced [79]. The opposite was observed in the low caloric group [79]. The link between caloric intake and autophagy regulation is interesting, as it highlights how energy deprivation (i.e., glucose and amino acid stress) can initiate protection via autophagy-mediated pathways to restore cellular energy supplies (Figure 2) [73]. In response to starvation, increased activity of the mitochondrial apparatus in turn increases mitochondrial ROS production, which activates autophagy [73]. Interestingly, a proteomics study in brain-dead rodents indicated that both metabolic changes and mitochondrial dysfunction are the two major canonical pathways affected in the kidney of brain-dead donors [80]. Even though no direct line of evidence connects these alterations to autophagy, it is nonetheless feasible that autophagy is indeed modulated in DBD donors.

A recent study from our group showed that the treatment of brain-dead rats with thyroid hormone T3, both a metabolic regulator and a powerful inducer of autophagy, improved liver function, while reducing apoptosis and oxidative stress [81]. In prosecution of this work [81], the protective effects of T3 preconditioning in brain-dead rats were indeed accompanied by an induction of autophagy in the liver, as evidenced by increased formation of LC3-II together with decreased (albeit nonsignificantly) levels of the autophagic substrate SQSTM1/p62 (Figure 4). Interestingly, in the kidney, T3 neither altered autophagy nor attenuated injury or apoptosis (Figure 4). Therefore, this suggests that T3-induced autophagy is not evident in the kidney and consequently, neither a reduction in apoptosis nor injury. In contrast, the reduction in apoptosis and injury markers in the liver does appear to be associated with increased hepatic autophagy, suggesting an important role for autophagy stimulation in the livers from brain-dead animals. We hypothesize that T3 boosts a transient peak in mitochondrial activity and ROS production, resulting in autophagy induction and subsequent protective effects during BD in the liver (Figure 5). This is supported by a recent study by Sinha et al. that showed that T3 induces autophagy via ROS-related pathways in the liver both *in vivo* and ex vivo [82, 83]. This phenomenon might not be evident in the brain-dead kidney due to extensive levels of oxidative stress [11], which might push the balance from a protective to a detrimental role for autophagy. This work therefore suggests that triggering oxidative pathways while simultaneously increasing autophagy could be an interesting strategy to improve liver function in DBD donors. In this respect, modulation of (mitochondrial) metabolism by compounds such as T3 might be the key to stimulation of transient ROS and subsequently protective autophagy activation (Figure 2).

3.3. Autophagy and Oxidative Stress during Preservation. Since most organs worldwide are still preserved on ice, several developments have been made to reduce ischemic injury during cold storage. The gold standard for preservation solutions in kidney, liver, pancreas and small bowel preservation is the University of Wisconsin (UW) storage solution [22, 84]. When comparing UW to HTK solution, the use of UW leads to decreased renal apoptosis and was linked to lower graft injury in human renal allografts [85]. UW contains, besides energy supplies (adenosine) and osmotic compounds, the antioxidants glutathione and allopurinol [84, 86] (Figure 2). However, the beneficial effects of these compounds are questionable considering their short life-spans and the fact that supplementation of glutathione to UW solution was unable to improve renal transplantation outcomes [84]. Nonetheless, a rodent study on kidney transplantation showed that treatment with hydrogen-rich saline (HRS), a novel antioxidant, immediately before ischemia attenuated transplantation-related renal injury and oxidative stress, while simultaneously inducing autophagic markers Beclin 1 and LC3-II [87]. Inhibition of autophagy with chloroquine attenuated these protective effects of HRS, suggesting that these effects were indeed autophagy-mediated and linked to oxidative stress [87]. Therefore, the addition of antioxidants to the preservation solution remains an interesting option to reduce graft injury (Figure 2). Conversely, prolonged cold ischemia of healthy kidneys in UW solution showed increased numbers of apoptotic and autophagic cells. In this case, the addition of bafilomycin A1 inhibited both autophagy and apoptosis [63]. These studies suggest that for the kidney the extent of cold ischemia injury could dictate whether either autophagy induction (in the case of shorter cold ischemia times) or inhibition (in the case of prolonged cold ischemia times) might be beneficial (Figure 2).

In the liver, cold storage of hepatic cells inhibited autophagic flux in both UW and Celsior solutions. However, only Celsior-stored cells reactivated autophagy upon reperfusion, while UW-stored cells displayed impaired lyso-autophagosomal fusion and elevated cell death [88]. Interestingly, pretreatment with simvastatin before cold ischemia led to the restoration of autophagy even in UW solution. This was accompanied by preserved cellular viability [88], suggesting possible benefits for hepatocytes when autophagy is induced during cold storage. These benefits might be linked to oxidative stress, as simvastatin-induced autophagy in cold-stored rat steatotic livers was accompanied by attenuation of oxidative stress [89], showing a link between ROS reduction and autophagy induction during cold liver preservation. In support of this, a study on liver transplantation in which the preservation medium (IGL-1) was enriched with trimetazidine, a fatty acid oxidation inhibitor, showed reduced oxidative stress yet activation of autophagy accompanied with reduced mitochondrial damage and hepatic injury [90]. These data therefore suggest that during cold storage of the liver, a strategy is preferred that simultaneously attenuates oxidative stress and stimulates autophagy.

The advantage of HMP over static cold storage has led to an increased implementation of this technique in the clinical setting. However, the lack of oxygen during HMP initiates high metabolic flux and subsequent high ROS production, particularly during the first minutes of reperfusion [91].

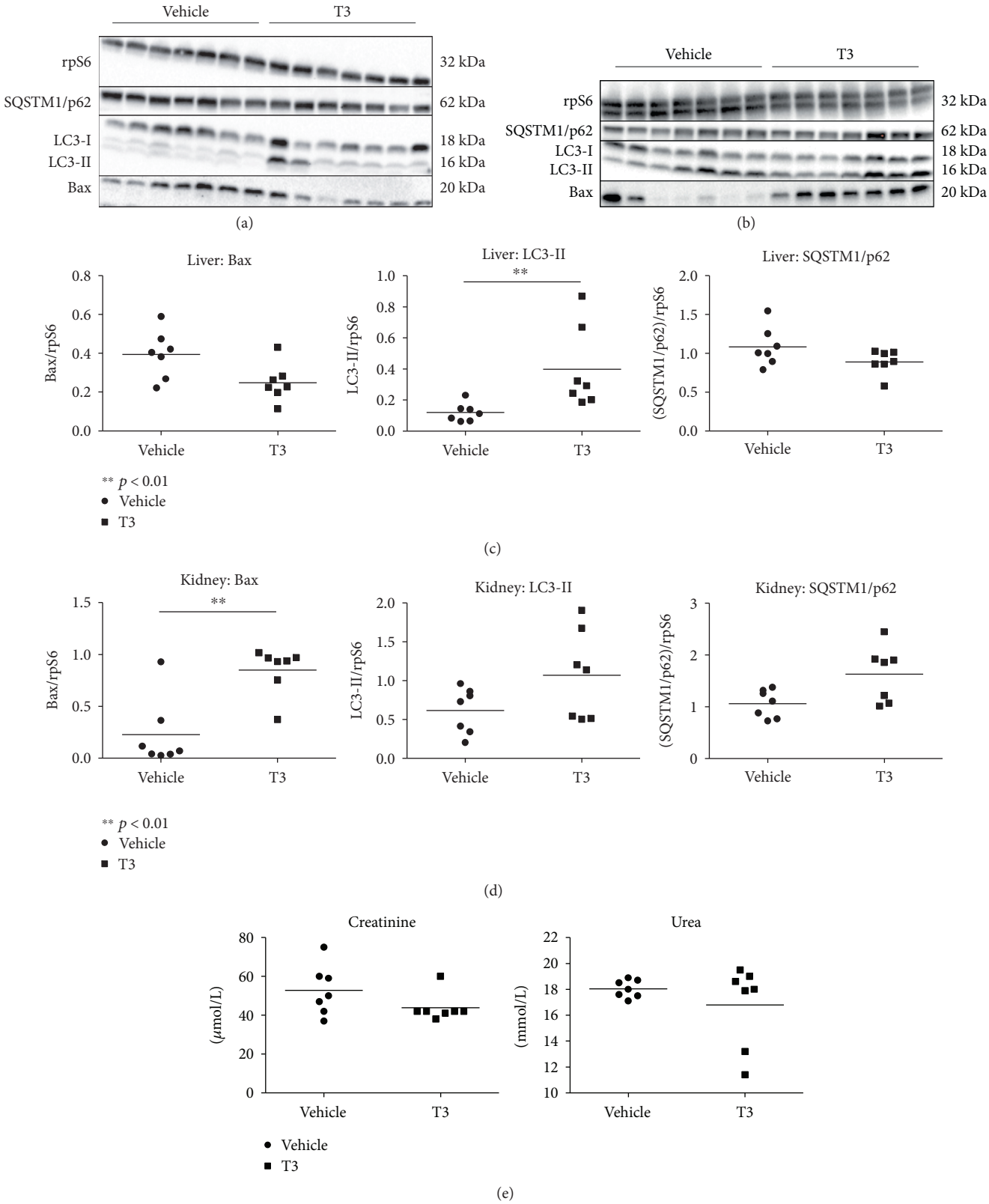

Figure 4: Posttranscriptional reduction of apoptosis and induction of autophagy in the liver of brain-dead rats following T_3 treatment, yet no effects in the kidney. Western blot expression of proapoptotic protein Bax and autophagy-related proteins LC3-II and SQSTM1/p62 in the liver (a, c) and kidney (b, d) and renal injury markers in plasma (e) of T3- or vehicle-pretreated brain-dead rats. Each lane represents an independent experiment. Results are presented as mean ± SD ($N = 7$ per group) ($^{**}p < 0.01$).

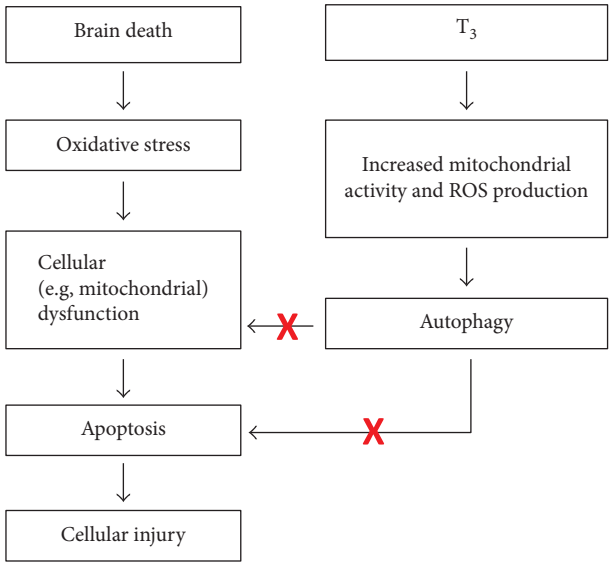

FIGURE 5: Proposed mechanism of T_3 preconditioning during brain death in the liver.

Therefore, benefits of oxygen addition to HMP solution are currently being investigated. Animal studies on liver and kidney HMP indicate that oxygenated HMP reduced mitochondrial flux and subsequent ROS production [91–93]. ROS production is further reduced when grafts are perfused at higher temperatures, such as subnormothermic (25°C) or normothermic (37°C) temperatures [94, 95]. Unfortunately, the effects of HMP on autophagy remain unexplored (Figure 2).

3.4. Autophagy and Oxidative Stress in the Recipient. As the extent of the oxidative response is dependent on ischemic duration, it is possible that the role of autophagy during IRI is dependent on the extent of oxidative stress. In this case, mild IR stress would trigger protective autophagy to counteract the oxidative damage experienced, for example, through autophagy-specific degradation of mitochondria (mitophagy). Regulation of mitophagy is thought to occur via oxidation of cardiolipin [29, 96–98], which plays a central role by mediating increased mitochondrial ROS release which serves as the signal for mitophagy initiation [99]. However, upon a very strong oxidative response (during severe reperfusion injury), an energy-dependent process as autophagy is not advised. In this case, autophagy stimulation could deprive much-needed energy, induce still elusive autophagy-dependent cell death mechanisms [100], or lead to the production of toxic lipofuscin (Figure 2) [74].

In the liver, IRI induced oxidative stress, autophagy, and apoptosis. These effects were attenuated by pretreatment with the antioxidant astaxanthin, potentially via modulation of the MAPK protein family [101]. In a study on renal IRI, increased oxidant production and autophagy markers were evident which suggests a tight and direct relationship between these two processes in which autophagy serves a protective role against renal oxidative damage [102]. In the heart, autophagic flux was analyzed during IRI in the presence of either H_2O_2 or the antioxidant N-2-mercaptopropionyl glycine (MPG). H_2O_2 increased, while MPG decreased autophagic flux, suggesting that autophagy is regulated by oxidative signals during cardiac IR. Interestingly, autophagy attenuation (by using heterozygous beclin $1^{+/-}$ mice) improved injury in this experimental setting [103]. Altogether, these findings imply that oxidative signals influence autophagy during IR and might be responsible for the exact role (protective or detrimental) autophagy plays in IRI, depending on the type of organ.

Several modulators of oxidative stress and autophagy have been clinically tested or are commonly used following transplantation. One method to combat the oxidative burst in recipients is ischemic postconditioning (IPoC), a technique that involves the temporal cessation of blood flow to a remote tissue such as a limb or locally via constriction of a nearby afferent artery. Despite promising preclinical results of local [104] and remote IPoC [105], clinical trials investigating both techniques did not report improved renal function after transplantation [104, 106]. Interestingly, IPoC also stimulates autophagy in the heart [107, 108], but this seems to depend on the postreperfusion time [109]. A clinical study on the addition of the antioxidant human recombinant SOD showed decreased rejection rates and improved survival following cadaveric kidney transplantation, despite mixed preclinical results [17, 110]. These results might be autophagy-dependent, as SOD overexpression resulted in attenuation of starvation-induced autophagy and apoptosis [111].

Many of the compounds given to transplant recipients as part of their immunosuppressive regimen also modulate autophagy. These include calcineurin inhibitors cyclosporine and tacrolimus, mTOR inhibitors sirolimus (rapamycin) and everolimus, mycophenolate mofetil (MMF), and corticosteroids [55, 112]. Cyclosporine is a known autophagy inducer that has now largely been replaced by tacrolimus as the treatment of choice in most European and American transplantation centers, mostly because of its proposed nephrotoxic side effects [112]. Interestingly, these side effects are thought to be related to increased autophagic clearance and autophagosome formation, phenomena possibly mediated via oxidative stress and apoptosis [92, 113]. Tacrolimus, on the other hand, was recently identified as an autophagy modulator that acts via activation of transcription factor EB, which in turn increases the expression of both autophagy and lysosomal genes [114, 115]. However, it is likely that this is associated with a mild, beneficial induction of oxidative stress [116]. The use of rapamycin as well as second generation mTOR inhibitors such as everolimus and deferolimus has been used as part of immunosuppressive therapy mostly for their ability to limit T-cell proliferation, but these compounds also influence autophagy by means of mTOR inhibition. However, the use of rapamycin in animal models of renal IRI and transplantation has yielded questionable results. Some studies suggest that rapamycin improves mitochondrial homeostasis and, subsequently, reduces ROS production and cellular senescence [46]. On the contrary, a study on renal transplant recipients suffering from DGF shows that patients who received rapamycin had significantly lower chance to resolve DGF [117]. Interestingly, combined

treatment with tacrolimus or mycophenolate mofetil (MMP), an immunosuppressive drug that is known to activate chaperone-mediated autophagy, enhanced the positive effects of rapamycin [55, 118]. The mixed effect of rapamycin treatment might be attributed to the amount of injury and subsequent extent of autophagy activation [55]. Finally, corticosteroids have been part of most postoperative and maintenance immunosuppressive regimens over the past years [119]. Interestingly, methylprednisolone (MP) both suppresses and stimulates autophagy in animal models [55]. This difference could be related to the extent of the preceding injury, or could be dose- and time-dependent, as MP treatment has opposite effects on oxidative injury when it is administered acutely (beneficial) or chronically (damaging) in rat lungs [120]. If and how MP affects autophagy modulation posttransplantation and whether this is influenced by oxidative signals remains to be elucidated.

4. Conclusion

Oxidative stress is an important component of the transplantation process as well as a known inducer of autophagy. Although the exact mechanism behind the complex reciprocal relationship between oxidative stress and autophagy is only beginning to be understood, it seems to play a major role in the different roles autophagy and oxidative signals seem to play during transplantation. The potential protective properties of autophagy and low levels of oxidative stress, yet detrimental effects when excessive activation occurs (Figure 2), make these two processes interesting therapeutic or diagnostic targets in each step of the transplantation process, while their tight interaction supports the possibility to target both pathways with only one compound. However, how these processes are preferentially modulated depends on the specific step in the transplantation process, the type of organ, the age and gender of the donor, the ischemic time, and other contributing factors. Therefore, future research should try and decipher the complex, intertwined relationship of oxidative stress and autophagy during the transplantation process. Finding the optimal balance between autophagic and oxidative processes is crucial for the optimization of cellular longevity and thereby graft survival.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

The Interrelation between Reactive Oxygen Species and Autophagy in Neurological Disorders

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Neurological function deficits due to cerebral ischemia or neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) have long been considered a thorny issue in clinical treatment. Recovery after neurologic impairment is fairly limited, which poses a major threat to health and quality of life. Accumulating evidences support that ROS and autophagy are both implicated in the onset and development of neurological disorders. Notably, oxidative stress triggered by excess of ROS not only puts the brain in a vulnerable state but also enhances the virulence of other pathogenic factors, just like mitochondrial dysfunction, which is described as the culprit of nerve cell damage. Nevertheless, autophagy is proposed as a subtle cellular defense mode against destructive stimulus by timely removal of damaged and cytotoxic substance. Emerging evidence suggests that the interplay of ROS and autophagy may establish a determinant role in the modulation of neuronal homeostasis. However, the underlying regulatory mechanisms are still largely unexplored. This review sets out to afford an overview of the crosstalk between ROS and autophagy and discusses relevant molecular mechanisms in cerebral ischemia, AD, and PD, so as to provide new insights into promising therapeutic targets for the abovementioned neurological conditions.

1. Introduction

Reactive oxygen species (ROS), an umbrella term for a category of active oxygen-containing compounds generated from aerobic metabolism [1], encompasses superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and free radical (superoxide and hydroxyl radicals). Each of these compounds can damage biomacromolecules essential for various cellular processes [2], while simultaneously playing an indispensable role in the redox signaling cascade required for critically important biological events [3]. ROS are likely to cause oxidative stress when the oxidation of ROS outweighs the antioxidation [4], which is believed to damage cells. Compared with other organs, the brain has the most active oxidative metabolism, with a high demand for oxygen. The brain's active oxidative metabolism combined with its deactivation of detoxification systems and severe deficiency of antioxidants jointly upsets the redox balance, causing immeasurable oxidative brain tissue injury. This process is closely correlated with the occurrence and development of cerebral ischemia and neurodegenerative diseases [5].

Autophagy is a precisely regulated biological process characteristic of eukaryotic cells during which the superfluous and damaged structures of cells are eliminated via lysosome degradation to maintain normal cellular physiological functions [6] for the purpose of adapting to all kinds of adverse stimuli. Existing studies suggest that autophagy is widely engaged in neuronal fate determination in diverse neurological conditions [7–9]. However, excessive autophagy can promote a programmed cell death, known as autophagic cell death or type II programmed cell death [10], which is morphologically distinct from apoptosis and necrosis. That is to say, moderate or optimal activation of autophagy is desirable, and neither excessive nor insufficient autophagy completely lacks toxicity to neurons [11].

ROS are associated with cell damage [12] and have traditionally been thought to function solely as unfriendly molecules, despite exposure to ROS being unavoidable for cells

in an aerobic environment [13]. However, an increasing number of researchers have found that ROS can participate in various physiological processes as a kind of signaling molecule, including the induction of autophagy that is considered to be an effective defense mechanism against cellular stress [14, 15]. More importantly, it is the mitochondrial ROS which is a master inducer of autophagy under conditions of nutrient starvation, ischemia, or hypoxia [16–18]. Conversely, activation of autophagy is a key part of the cellular response to oxidative stress because the process disposes defective components before further damage/aggregation occurs [19]. In summary, the interaction and the balance between ROS and autophagy can be a key part of regulating cellular homeostasis.

It is well established that both ROS and autophagy are strongly associated with neurological diseases, but clarifying the functional relationship of the two mechanisms seems to be difficult because of their dual role in many disease processes. This review is designed to state the role of ROS and autophagy in neurological disorders and their underlying molecular mechanisms so as to offer novel strategies for the treatment of nervous system diseases.

2. Reactive Oxygen Species (ROS)

2.1. Generation and Scavenging of ROS. It is now well documented that mitochondria are the main source of intracellular ROS; 90% of which are derived from the respiratory chain on the mitochondrial inner membrane. The generation of mitochondrial ROS is initiated by the formation of O_2^- via the combination of electrons leaking from the mitochondrial respiratory chain complexes (mainly complexes I and III) and O_2. Highly active O_2^- can then be transformed into more stable H_2O_2 in the presence of superoxide dismutase (SOD). The quick conversion of H_2O_2 into H_2O can be catalyzed by catalase (CAT) and glutathione peroxidase (GSH-Px) and serves as the source of OH^- as well [3, 20, 21].

Under normal circumstances, ROS emissions in mitochondria are rather low and render minimal damage because mitochondria have potent antioxidant defense systems that sufficiently scavenge unneeded ROS. Whereas, unbridled ROS ensue only if mitochondria are subjected to deleterious incidents while simultaneously experiencing a drop in transmembrane potential. There is a positive-feedback mechanism called "ROS-induced ROS release" (RIRR) that accounts for the interaction between ROS and mitochondria. During RIRR, a burst of mitochondrial ROS is evoked by ROS, reducing mitochondrial membrane potential (MMP) and causing a longer opening of mitochondrial permeability transition pores (mPTP) [22]. Generally, moderate activation of mPTP is required for healthy mitochondrial metabolism. Once mitochondria are attacked by an inappropriate release of ROS, mitochondrial membrane depolarization interferes with mitochondrial respiratory chain function and can create a vicious circle provoking further ROS accumulation [23].

As mentioned, abnormally high levels of ROS can be quickly neutralized to cellular levels by a complex network of various robust antioxidants, which is essential for sustaining the normal functions of cells [24]. There are two major antioxidative systems that consist of enzymatic and nonenzymatic antioxidants. The former is represented by SOD, CAT, and GSH-Px, while the latter includes glutathione (GSH), vitamin C, vitamin E, and so forth. Nevertheless, when there exists any redox imbalance between the generation and the scavenging of ROS, oxidative stress occurs, leading to unpredictable oxidative damage to organelles, proteins, lipids, and DNA, as well as the disruption of cellular structures and functions and eventually cell death [25] (Figure 1).

2.2. Biomarkers of ROS/Oxidative Stress. Due to the nature of ROS, which are active for a relatively short lifespan, various complex and time-consuming detection means such as electron spin resonance and spin trapping technology are relatively difficult to practically implement, and the results are also often offset by the mixing of heterogeneous groups [26]. Therefore, ROS or oxidative stress level is usually measured by monitoring the activity of cellular antioxidant enzymes such as SOD and GSH-px, each of which can indirectly reflect the ability to remove ROS [27]. Concurrently, GSH and malondialdehyde (MDA) are used to mirror oxidative stress resistance and injury.

Superoxide dismutase, a copper-containing protein isolated from bovine red blood cells by Mann et al. for the first time in 1938, was rediscovered and named as SOD by Fridovich and Mccord in 1969. SOD is able to scavenge ROS. The glutathione peroxide (GSH-Px) is extensively present in the cytoplasm. Mitochondria contain two kinds of GSH-Px, GSH-Px1 and GSH-Px4, by which lipid peroxide induced by OH^- can be decomposed into the corresponding alcohol or peroxide-induced injury can be reduced [28]. As is widely known, common ROS involved in cellular damage are mainly OH^-, H_2O_2, and O_2^- [29]. The dynamic conversion among the three parts depends upon SOD and GSH-Px, only by which can O_2^- be reduced into H_2O, thus mitigating oxidation damage [30]. In conclusion, the activity of these two antioxidant enzymes may be the reflex of the ability to eliminate ROS [31] (Figure 1).

Glutathione (GSH) is the most abundant nonprotein thiol and broad-spectrum antioxidant in mitochondria and contains two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). The former accounts for about 95% of GSH and, as the primary ROS scavenger, can effectively remove H_2O_2 and O_2^- and other free radicals, while concurrently being transformed into recyclable GSSG via glutathione reductase.

In the above processes, catalase (CAT) and glutathione reductase (GR) are typically used in combination with SOD, GSH-Px, and GSH as potential antioxidant biomarkers to evaluate oxidative stress.

There are also some other ROS measurement parameters based on oxidation of lipids, proteins, and DNA. Some examples of these parameters are MDA, 4-hydroxy-2-nonenal (4-HNE), 3-nitrotyrosine (3-NT), and 8-OHdG. Any accumulation of oxidation byproducts implies deterioration through oxidative damage, but different byproducts represent the different levels of cellular damage. Excess ROS inflict irreversible damage to nucleic acids, which has been reported to be an early event in oxidative damage.

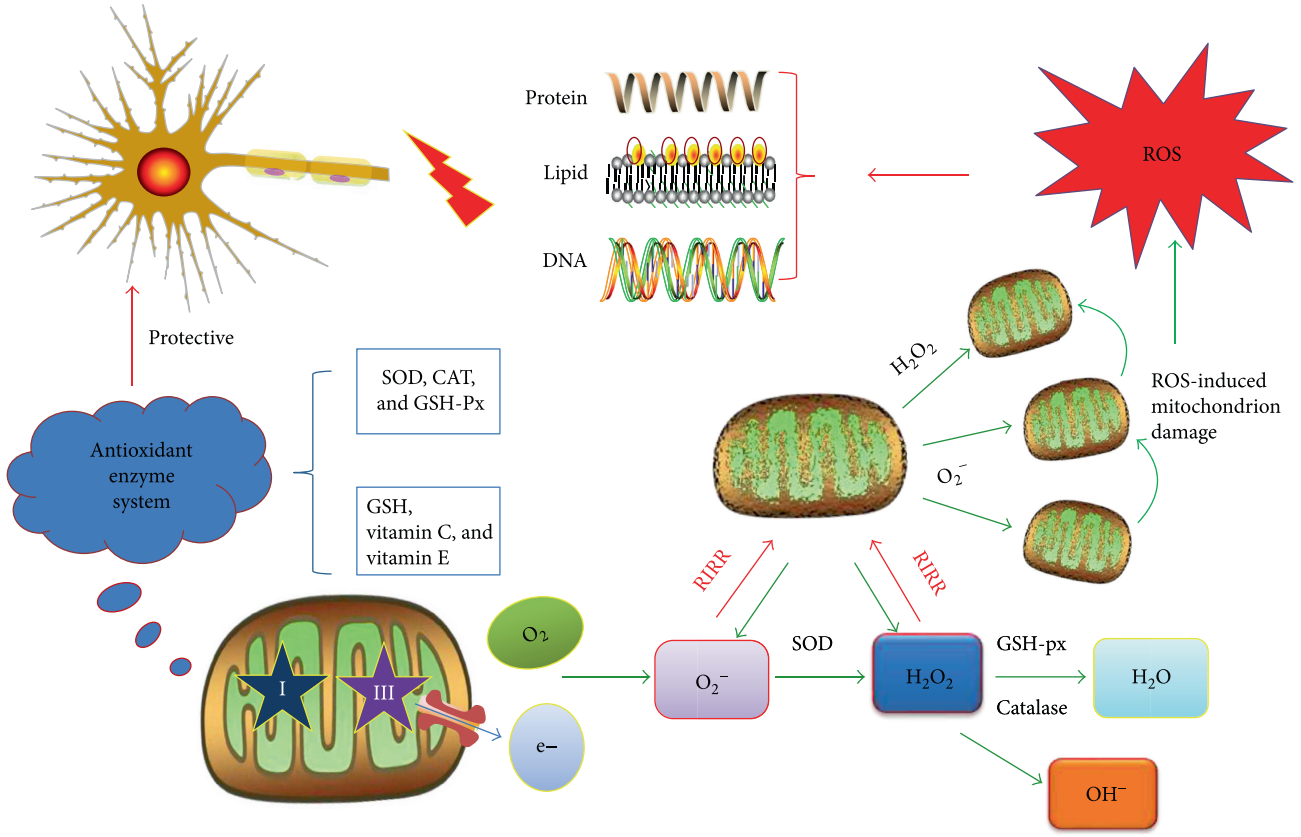

Figure 1: The generation and scavenging of reactive oxygen species (ROS) in mitochondria. The "→" refers to activation or induction, and the "⊢" refers to inhibition. Under normal or stress conditions, ROS is mainly born from the mitochondrial respiratory chain with the beginning of O_2^- production, followed by the conversion to H_2O_2 then OH^- under the catalysis of SOD and GSH-px. Defective mitochondria can instigate ROS accumulation with a "RIRR" positive-feedback mechanism. Excessive ROS can inflict severe damage on biomacromolecules, which can be counteracted by the antioxidant enzyme system to some degree.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a biological index generated by the oxidation of DNA along with the loss of its integrity. As a pivotal biomarker of endogenous oxidative DNA, 8-OHdG levels have been commonly assessed to estimate ROS-induced DNA lesions in multiple neurological disorders.

As for malondialdehyde (MDA), the ubiquitous final product of lipid peroxidation created by ROS attacking unsaturated fatty acids of biological membranes [32], its accumulation can incur cross-linking polymerization of macromolecules such as protein and nucleic acid, permeability and destruction of membrane structures, and eventually cell death. The degree of lipid peroxidation can be estimated by the quantity of MDA in the tissue, so MDA is proposed to be one of the indicators of intracellular oxidative stress [33]. Similarly, 4-hydroxy-2-nonenal (4-HNE), a specific clinical detection index of polyunsaturated fatty acid peroxidation, is also currently utilized to measure the extent of oxidative lipid damage. Isoprostane is the best available index of lipid peroxidation because of its stability. In summary, these indicators may better assess oxidative damage of brain tissue because various polyunsaturated fatty acids are susceptible to ROS during oxidative metabolism in the brain.

Likewise 3-nitrotyrosine (3-NT), an important metabolite of oxidative lesions in protein, has been measured in brain tissue, with increased levels in PD and AD populations. 3-NT is stable both in vitro and in vivo. There is a lot of value in the assessment of oxidative stress for clinical research [34].

2.3. ROS/Oxidative Stress-Related Signal Pathway. Keap1/Nrf2/ARE cascades have proven to be the most important antioxidant defense, and almost all protective antioxidant genes contain antioxidant response elements (ARE). When exposed to oxidative stress, Keap1 (kelch-like ECH-associated protein 1) can be separated from Nrf2 (nuclear factor erythroid 2-related factor 2) by uncoupling activity or reduction of ubiquitination and degradation, then translocation of Nrf2 into the nucleus targeting ARE. Nuclear translocation leads to the expression of antioxidants and phase II detoxifying enzymes, which is shown to greatly reduce ROS and ensuing oxidative damage.

In addition, several intracellular signaling pathways related to redox state, such as PI3K/Akt, JNK, MAPK, and ERK, can dissociate Nrf2 from Keap1 through phosphorylation of Nrf2. These signaling pathways also cause Nrf2 to translocate to the nuclease and activate the antioxidant

system, which is expected to augment the oxidative defense capacity [35, 36].

2.4. Reactive Oxygen Species (ROS) in Neurological Disorders. Loss of neurons is a key link in the pathophysiological process of nervous system diseases, which is mediated by oxidative stress, mitochondrial disturbances, abnormal protein aggregation, and so on [37]. One of the most important problems is oxidative stress, or ROS [38]. To our knowledge, the brain weighs just 2% of the body's weight, but its metabolic oxygen consumption accounts for 20% of total oxygen consumption of the organism under nonstress conditions. High oxygen demand is always accompanied by more ROS. The brain is rich in various polyunsaturated fatty acids sensitive to ROS, but is relatively devoid of antioxidant enzymes and GSH, adding that neurons are considered terminally differentiated cells [39], which make brain tissue more inclined to suffer damage from ROS [40, 41].

Robust evidence suggests that ROS display a recognized role in neuronal death after brain ischemia [42]. Either an initial burst of ROS induced by ATP consumption and mitochondria depolarization in the ischemic phase or the Ca+-dependent ROS generation at the reperfusion stage can pose a hazard for neurons [42]. As noted earlier, excessive production of ROS can not only damage cellular macromolecules but also impair antioxidant enzymes and nonenzymatic antioxidants during I/R insult, which is unfavorable for neurofunctional recovery. Sharma and Airao have shown that lipid oxidation byproducts such as MDA are markedly increased in ischemic tissues, but SOD, CAT, and GSH levels are reduced. Early administration of solasodine can ameliorate progressive ischemic injury through its potent antioxidant properties [43]. The Nrf2/ARE pathway is referred to as a potent defense mechanism against oxidative stress, which is expected to be a feasible direction of antioxidant treatment against ischemia-reperfusion (I/R) injury. In the Shah and Li study, they found that Nrf2 knockout mice in the I/R group present more obvious neurologic deficits than the wild type group with a significant increase in the area of infarction [44]. Enhancing the activation of Nrf2 by tBHQ, a natural Nrf2 inducer, can reduce and limit brain damage and is therefore possibly a practical prevention strategy for stroke-prone patients [45].

Studies have shown that the cellular damage in the early stages of AD is ascribed to oxidative stress [46], and notably, a large number of markers of oxidative stress are located in intracellular NFTs, a hallmark of the brains of AD patients [47]. A significant decrease in GPx and CAT activities and total GSH levels, which indicates a feeble antioxidant defense system in early AD, may facilitate the development of the disease. Meanwhile, extensive experiments collectively verify that antioxidants do delay the occurrence and progression of AD [48]. These oxidative stress indicators are used to characterize the earliest events of AD and are reliable tools for early diagnosis and prevention of AD [49].

Recent progress in PD has revealed that dopaminergic neurons are susceptible to oxidative stress because of inherent biological features. Clear evidences show that 4-HNE within such body fluids as CSF and serum is widely described as a clinical parameter of oxidative damage in PD individuals. Reactive (OH) and subsequent MDAs have been reported to be significantly increased in PD patients, which contribute to dopaminergic neuronal loss [50]. Nrf2 exists in the nigral dopaminergic neuron cytoplasm, but is located in the nucleus of age-matched PD patients, which strongly suggests that Nrf2 may contribute to combating oxidative brain damage via the transcription of genes encoding antioxidant enzymes [51]. Recent studies have claimed that upregulation of Nrf2 provides neuroprotection against oxidative stress-induced neurotoxicity in PD. Rb1 can enhance the transcriptional activation of Nrf2 and upregulate the expression of HO-1, an endogenous antioxidant enzyme and downstream effector of Nrf2, by modulating PI3K-mediated Nrf2-ARE signal pathway, which is shown to serve as a rational cytoprotective agent against oxidative insults of dopaminergic cells [52]. Taken together, ROS elevation initiates neuronal damage and we propose that Nrf2-related agents look set to offer an up-and-coming clinical therapy.

3. Autophagy

Autophagy was observed in mouse hepatocytes by Ashford and Porter for the first time in 1962 and visually described as cellular self-eating [53]. Nevertheless, it was De Duve that first came up with the concept of autophagy in 1967 [54]. Autophagy refers to macroautophagy in this review, the most common and well-studied form, which is distinguished from microautophagy and chaperone-mediated autophagy (CMA) by the different degradation pathways of substrates [55, 56]. Autophagy induction is a complicated and ordered multistep process, which mainly includes the following steps: the signal stimulus, then autophagosome formation and fusion with lysosomes, and finally the degradation and release of its contents.

It has also been copiously reported that autophagy can facilitate the renewal of cellular constituents to guarantee energy and materials of quality needed to sustain metabolic reactions, which orchestrates such biological processes as proliferation and differentiation of cells under various physiological or pathological conditions [57]. Typically, autophagy exists at a low level and a basal rate in most cells [58], but it can be activated rapidly in response to excessive release of ROS, abnormal aggregates of misfolded proteins, or a collapse of mitochondrial membrane potential (MMP) apart from infection, cancer, ATP, or nutrient deficiency [59, 60].

It is well established that only adequate autophagy is a kind of cellular self-defense mechanism in times of oxidative stress and other unfavorable conditions [61]. However, improper autophagy above or below a certain threshold is instead disadvantageous [11], likely accelerating the progression of all the related diseases such as neurodegenerative diseases, cerebral ischemia, and cancer [62, 63].

3.1. Autophagy-Relative Marker Proteins. As discussed previously, autophagic elimination is a highly sophisticated process during which unwanted or redundant organelles and bits of cytoplasm are enveloped then sweeped away in a lysosome-dependent manner. Each step is finely regulated

by relevant proteins that were first discovered in yeast but later verified in higher organisms [63, 64].

LC3 is a mammalian, homologous protein of Atg8 in yeast that has been identified to be the most widely used specific marker of autophagy initiation. LC3 is first synthesized as its precursor, then cut up into its cytosolic form, LC3-I, which can be processed into LC3-II [65]. LC3-II specifically binds to the newly formed autophagosome essential for the elongation stage of the phagophore membrane. The amount of autophagosome can be mirrored by the expression of LC3-II or LC3-II/LC3-I [66]. Mizushima et al. [67] were able to dynamically trace the formation of autophagosomes by using fluorescence characteristics of GFP in established GFPLC3 transgenic mice, which greatly facilitated the study of the molecular mechanisms of autophagy. Beclin1, the first mammalian autophagy-related gene to be identified, regulates the activity of autophagy particularly in the initiation phase by combining with different ligands [68]. Beclin1 can modulate autophagic flux by interacting with PINK1 [69].

In addition, there are observable changes of p62/SQSTM1 in the progression of canonical autophagy [70]. P62 is negatively correlated with autophagy activity, reflecting the degradative capability of autophagy and the intensity of autophagic flux [71]. The receptor protein p62 can be recruited to the autophagosome membrane when LC3-interacting region (LIR) motif targets a substrate (ubiquitinated protein aggregates, damaged mitochondria [72]) and initiates selective degradation in an autophagy-lysosome manner.

3.2. Autophagy-Relative Signal Pathway. Prevailing studies indicate that signal transduction pathways associated with autophagy may be more complex than the following two: the mammalian target of rapamycin (mTOR) pathway and the class III phosphatidylinositol 3-kinase (PI3K-III) complex.

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is engaged in autophagy modulation as a dominant downstream negative regulator [73]. mTOR complexes exist in two types, namely mTORC1 and mTORC2, which are distinguished by different components. mTORC1, a regulatory associated protein composed of Rictor, has been demonstrated to terminate the autophagy progression as a critical signaling molecule that is susceptible to the strong inhibition of rapamycin [74, 75]. When cells suffer hypoxia, energy depletion, and other stimuli, mTORC1 activity is simultaneously restrained with the activation of autophagy. Suppressed mTORC1 plays a causal role in the activation of ULK1 complex by dephosphorylating the autophagy-related gene13 (Atg13) and mediating a tighter combination of ULK1, Atg13, and FIP200. ULK1 is homologous with Atg1 in yeast, which has been found to be involved in the induction of autophagy. The ULK1-Atg13-FIP200 complex is not only a direct target of mTOR but a key regulator of other autophagy-related signaling pathways.

The PI3K-III complex is composed of VPS34 (catalytic subunit), Beclin1, and Atg14. When activated by the ULK1 complex, the PI3K-III complex is positioned into the endoplasmic reticulum and further generates PI3P that binds to downstream effectors, playing an important role in the earlier period of autophagic vacuole formation [76, 77]. When discussing the PI3K-III complex, it is common to mention that the class I PI3K and its downstream target AKT, as with MAPK/ERK1/2 signaling, which can exert negative regulatory effects at any stage of induction of autophagy via activating mTOR [78].

Arguably, distinct signaling pathways involved in the autophagic process vary with different adverse stimuli. AMP-dependent protein kinase (AMPK), an upregulated modulator of autophagy, can sense subtle levels of ATP. On the one hand, AMPK can activate autophagy with a direct inhibitory effect on mTORC1 [79]. On the other hand, p-AMPK can activate TSC1-TSC2 complex, indirectly suppressing the activity of mTORC1 and concurrently initiating autophagy [80]. In addition, AMPK can also combine with ULK1 complex and phosphorylate ULK1, accelerating the progress of autophagic membrane formation [81] (Figure 2).

3.3. Mitophagy. Past studies have argued that autophagy does not select which substrates are to be degraded [82]. However, a widely accepted view, proposed in 2005, is that there is a selective form of autophagy in which damaged or unnecessary mitochondria are eliminated [83]. This nonclassical autophagy was defined as mitophagy, and simultaneously or successively, other types of selective autophagy such as xenophagy, pexophagy, ribophagy, and reticulophagy were also identified [59, 84].

Mitochondria are a sensitive organelle ubiquitously found in eukaryotic cells. They are responsible not only for energy-generating processes, but also for producing a basic amount of ROS [85]. Mitochondria form a complicated network regulated by other cellular mechanisms, in which mitochondria are interconnected and interlocked in a perfectly coordinated order. Impaired mitochondria are a threat to proper cellular function because they result in a lack of energy generation and excessive release of ROS [86]. Therefore, it is urgent that dysfunctional mitochondria that interfere with the energy supply and provoke oxidative stress be quickly removed [87]. Fortunately, mitophagy can shoulder this responsibility as an effective cytoplasmic protection mechanism.

Mitophagy is a programmed mitochondrial elimination mechanism that fosters a balance of mitochondrial quantity and quality [59, 87]. It usually occurs in the case of an abnormal increase of ROS, poor nutrition, hypoxia [88], cells senescence, and such stress. These stimuli can cause mitochondrial membrane depolarization or a loss of MMP. Pathological opening of the mPTP may serve as the switch for mitophagy. Existing studies suggest that there are two relatively recognized mitophagy pathways involved in mitochondrial homeostasis. These two pathways are the PINK1/Parkin-mediated pathway and the Bnip3/Nix-mediated pathway. The PINK1/Parkin-mediated pathway is closely associated with Parkinson's disease and is a topic of current research [89].

3.3.1. PINK1/Parkin-Mediated Pathway. PINK1, a serine/threonine protein kinase, is located on the outer membrane of mitochondria and is the upstream regulator of Parkin [90]. Parkin is an E3 ubiquitin ligase, which is present in

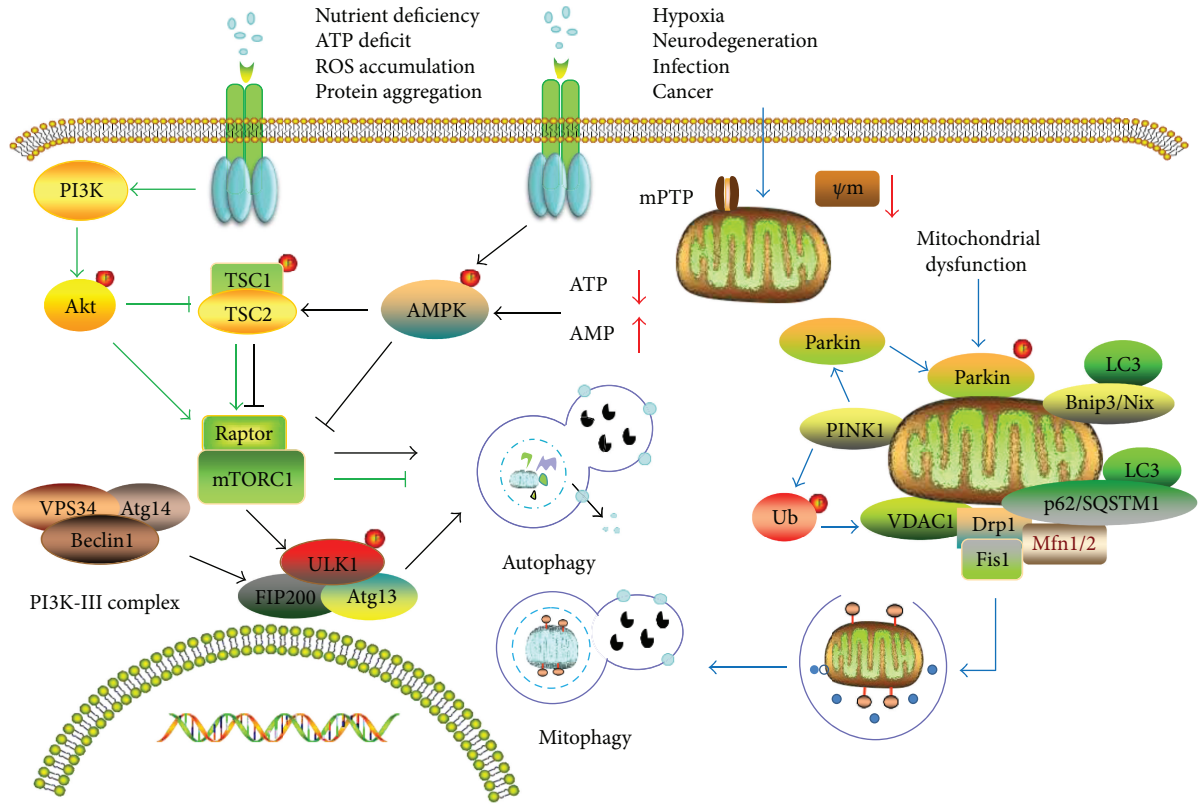

FIGURE 2: The generation and scavenging of reactive oxygen species (ROS) in mitochondria. The "→" refers to activation or induction, and the "⊢" refers to inhibition. Under normal or stress conditions, ROS is mainly born from the mitochondrial respiratory chain with the beginning of O_2^- production, followed by the conversion to H_2O_2 then OH^- under the catalysis of SOD and GSH-px. Defective mitochondria can instigate ROS accumulation with a "RIRR" positive-feedback mechanism. Excessive ROS can inflict severe damage on biomacromolecules, which can be counteracted by the antioxidant enzyme system to some degree.

the cell plasma [91] but has no mitochondrial targeting sequence (MTS) [92]. As a matter of fact, PINK1 can be degraded away quickly by proteolytic enzymes in healthy mitochondria. In the disturbed mitochondria, it will accumulate following depolarization of the membrane potential, phosphorylate Parkin, and then recruit Parkin from the cytoplasm [90]. Along with strengthening E3 ubiquitin ligase activity, Parkin can ubiquitinate the mitochondrial matrix proteins (voltage-dependent anion-selective channel protein 1, VDAC1), recruit p62/SQSTM1 to the surface of mitochondria, and then combine with LC3 to initiate mitophagy [93].

Emerging research indicates that RAD6A (Ube2a), a gene encoding ubiquitin binding enzyme (E2) that is required for the ubiquitination and subsequent clearance of defective mitochondria, can operate with Parkin to regulate mitophagy upon mitochondrial depolarization in mice cortical neurons. Whether the program is dependent on PINK1 needs further scrutiny [94].

3.3.2. Bnip3/Nix-Mediated Mitophagy. Bnip3, a proapoptotic protein, has some degree of homogeneity with BCL-2. Nix is 56% homologous with Bnip3. Both widely existed in mitochondria and are implicated with autophagy and mitophagy in particular [95]. Bnip3 induces autophagy after hypoxic damage and has been reported to have a protective effect by removing injured mitochondria [96]. Recent studies have shown that Bnip3/Nix directly interacts with LC3 to activate the mitophagy pathway [97, 98]. Some researchers believe that though Bnip3 and Nix are involved in mitophagy upon the loss of mitochondrial membrane potential, they may execute mitochondrial clearance via independent but functionally related mechanisms [99, 100] (Figure 2).

Additionally, Mieap can also induce mitophagy after ROS and oxidative damage to restore a healthy pool of mitochondria [101]. Last but not least, mitochondrial fusion, division, and transportation are tightly linked to mitophagy [102].

3.4. Autophagy in Neurological Disorders. Not surprisingly, autophagy is extensively observed in nervous system disorders [61]. It has long been thought that autophagy is the primary means for the biodegradation of abnormal protein aggregation and dysfunctional organelles in CI, AD, and PD [103]. Defects in mitochondrial autophagy will aggravate ischemic tissue damage with irreversible neurologic deficit [104], render cognitive and memory defects in AD as a consequence of progressive aggregation of Aβ [105], and promote dopaminergic neuronal death and the occurrence of PD [106]. These results of defects in mitochondrial autophagy indicate that mitophagy acts as an endogenous protective mechanism in the process of neurological disorders. At present, although a growing number of studies have argued that autophagy is activated in various rat and mouse models of

cerebral ischemia or hypoxia-ischemia [9, 107–110], whether autophagy is protective or detrimental in the process of CI still remains unclear [111].

4. Reactive Oxygen Species (ROS) and Autophagy

A growing body of reports has demonstrated that most stressful events, such as nutrient deficit and hypoxia, which necessitate a greater energy supply and then aggravate mitochondrial burden along with increasing ROS, are related to the initiation of autophagy [16]. Intriguingly, an increasing amount of evidence suggests that ROS are seen as essential signals to activate autophagy under various stimulating conditions [112, 113]. Both moderate and increased ROS levels can specifically trigger mitophagy which is conducive to cell survival in a different manner, while only excessive ROS can activate general autophagy [114].

The molecular signaling pathways involved in both the initiation and execution of autophagy following exposure to ROS are sophisticated [16, 18]. The pathways mainly include transcriptional progress in the nucleus and posttranscriptional progress in the cytoplasm. These specific transcriptional regulatory mechanisms first involve the activation of HIF-1, p53, FOXO3, and NRF2; then, the corresponding proteins are produced and modulation of autophagy occurs where the cytoplasm was exposed to ROS. Take hypoxia-inducible factor (HIF) for example, it is involved in cell survival under hypoxic conditions and participates in the transcription of Bnip3 and NIX in response to ROS. These autophagy-associated protein products can constitutively stimulate autophagic clearance of damaged mitochondria and decrease ROS levels [115].

In addition, numerous studies have supported that ROS may regulate autophagy via mTOR-dependent pathways in the cytoplasm [116–118]. Nevertheless, most of the literature maintains that ROS available to elicit autophagy are mainly H_2O_2 and O_2^- produced by mitochondria [14, 112]. When there is an elevated level of H_2O_2, a relatively stable and prolonged stimuli, suppressed autophagy via the PI3K-Akt pathway, can be reactivated by blocking PTEN as well as inhibiting the activity of Akt or mTORC1 [119]. Similarly, H_2O_2 in excess can induce autophagy in an AMPK-dependent manner and is accompanied by the decline of mTORC1 activity [18]. Beyond that, a wide range of stress response proteins such as p38MAPK, extracellular regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) is also involved with autophagy induction in the presence of abundant ROS [120]. Taken together, it is an indisputable fact that ROS is an available regulator despite autophagy making a difference in both cell survival and death as a double-edged sword.

From another perspective, autophagy has been proposed as a potential survival mechanism in the face of ROS production by removing damaged or redundant components to prevent unnecessary oxidative damage [19]. Furthermore, there are increased intracellular ROS levels in cells with defective autophagy protein Atg7 [121]. Specially, the selective elimination of dysfunctional mitochondria via autophagy also serves as a cytoprotective process to limit the production of ROS and avoid potential oxidant injury [122].

It is also believed that a number of signal transduction pathways related to autophagy are available to modulate ROS. The Keap1-Nrf2 system is now considered a defense mechanism upon exposure to oxidative stress [123, 124]. As mentioned earlier, the p62/SQSTM1 protein, or p62 for short, may contribute to autophagosome formation as an autophagic adaptor and/or receptor [125]. Phosphorylation of P62 in the mTORC1-dependent autophagy pathway can promote the integration of ubiquitinated cargos and phosphorylated Keap1, which is necessary for the degradation of Nrf2 [126, 127]. Released Nrf2 is reactivated, translocated into the nucleus while binding to ARE, and eventually stimulates transcription of antioxidant genes. Beyond that, mitochondrial hexokinase II (HKII) shares a deep relationship with autophagy and redox homeostasis. HKII induces the inactivation of mTORC1, further opens mPTP, and creates a preventive antioxidant defense by decreasing release of ROS [128, 129].

In conclusion, there is little doubt that ROS play a positive role in the activation of autophagy under various stimulating conditions [112, 113]. By coincidence, autophagy plays a crucial role in maintaining redox homeostasis [6]. ROS can induce autophagy, and autophagy serves as a buffer system to control the level of ROS in cells and reduce their toxic effects [130]. The interplay of autophagy and redox response via various signaling pathways may be involved with the modulation of cellular homeostasis [127] (Figure 3).

4.1. Reactive Oxygen Species (ROS) and Mitophagy. As stated earlier, mitochondria are believed to be the primary source of ROS. Coincidentally but unfortunately, they are also the major target of oxidative stress triggered by ROS, which may result from the fact that mitochondria are an important site for nucleic acid, lipid, and amino acid production. Excessive ROS stimuli can inflict peroxidation damage on these biomacromolecule precursors and create toxic byproducts [131]. Note that mtDNA lacks the protection of histones, and its repair capacity is rather poor. It is therefore more vulnerable to ROS than nuclear DNA [132] and is bound to leave mitochondria heavily damaged by ROS.

Mitochondrial dysfunction caused by a high concentration of ROS not only can activate and regulate nonselective autophagy, but also can be involved in mitophagy which selectively removes damaged mitochondria. ROS and oxidative stress have been shown to be involved in the recruitment and localization of Parkin and DJ-1, specific proteins that are closely tied to the activation of mitophagy [133].

Selective autophagy is a protective mechanism that reduces ROS production by means of removing unneeded mitochondria, thereby alleviating oxidative damage [16, 122]. More importantly, defects in mitophagy can aggravate lipotoxicity, hinder selective degradation of defective mitochondria caused by ROS, and thus cause subsequent damage to the cells [134].

Haddad et al. [94] discovered that RAD6A can cooperate with Parkin to ubiquitinate mitochondrial proteins associated with the initiation of mitophagy for clearing dysfunctional

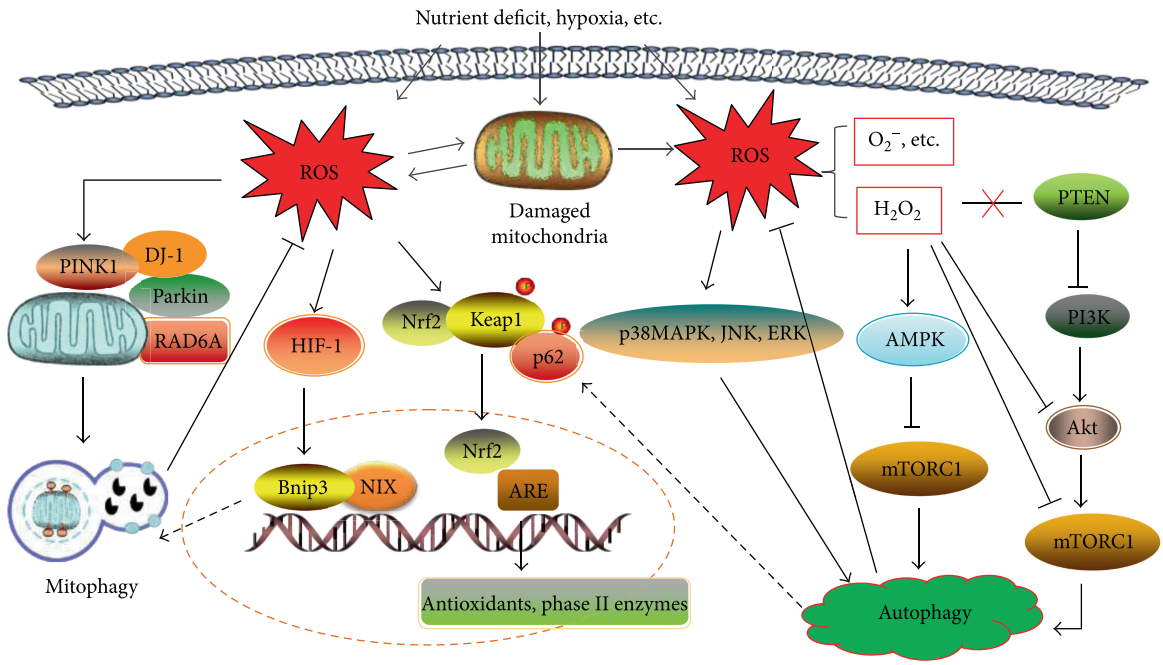

Figure 3: The interrelation of ROS and autophagy/mitophagy, coupled with the relevant signal transduction pathways. ROS available to induce autophagy is mainly mitochondrial H_2O_2 and O_2^-, which may modulate autophagy via mTOR-dependent pathways. ROS-induced autophagy and mitophagy both can abort ROS for redox homeostasis. In response to abundant ROS, the Keap1/Nrf2/ARE cascade is activated as a potent antioxidant mechanism. Phosphorylation of P62 by autophagy can promote the integration of phosphorylated Keap1 and ubiquitinated Nrf2, then negative regulation of Keap1 frees Nrf2 from degradation, and reactivated Nrf2 is translocated into the nucleus to bind to ARE for the transcription of antioxidant genes and phase II enzymes.

mitochondria and dampening oxidative stress. Particularly, RAD6A mutations cause neuronal function defects primarily by disrupting mitophagy (Figure 3).

4.2. Reactive Oxygen Species (ROS) and Autophagy (Mitophagy) in Neurological Disorders. ROS are described as the culprit of almost all neurological conditions [135]. Mounting evidence has indicated that ROS participate actively in autophagy in many cells, including neurons [131, 136]. Autophagy removes or degrades nonfunctional cytoplasmic content as an intracellular self-purification mechanism. Neurons are highly sensitive to autophagic degradation, and the integrity of mature neurons depends on the high level of autophagy because of their postmitotic nature [14, 137]. Also, autophagy can reduce ROS damage by eliminating unnecessary or damaged organelles and abnormal protein aggregates, as well as inhibiting the excessive activation of ROS in response to neuronal damage, which is conducive to the survival of nerve cells [138]. Emerging evidence indicates that autophagy may exhibit an antioxidant defense system, which has been proposed to provide a remarkable impact on neuronal bioenergetic health [139].

4.2.1. Reactive Oxygen Species (ROS) and Autophagy (Mitophagy) in CI. Ischemic cerebrovascular disease (CI) is a leading cause of death and disability worldwide [140]. Currently, endovascular intervention and venous thrombolysis are conventional therapies for restoring the blood supply required for the recovery of nervous function. However, both animal studies and clinical findings have revealed that reperfusion following ischemia results in a more serious brain damage [141]. The ischemia-reperfusion injury is a complicated pathological process involving multiple factors, among which oxidative stress stands out [5, 142]. There is an extensive damage of mitochondria, including irregular mitochondria swelling and their crista fragmenting, in CI especially during the acute phase. This damage stimulates mPTP to open continuously, leading to a change in membrane potential, energy deficit, and ROS generation, thus inducing autophagy.

It has been observed that autophagy occurs dramatically in the mouse striatum and cortex following cerebral hypoxic-ischemic injury and can then be strongly amplified by an ensuing overproduction of ROS. Autophagy in this context can substantially rescue neurons in the ischemic penumbra by preventing necrosis and apoptosis via eliminating impaired mitochondria [143].

It has been reported that FA deficiency dramatically alters ischemia-induced activation of autophagy. This is reflected by the elevated levels of LC3 and Beclin1 expression, which are accompanied by a remarkable increase in 8-OHdG, indicating that FA deficiency may enhance autophagy levels by triggering oxidative damage [144]. One study has shown that both ROS and autophagy are engaged in reperfusion injury after cerebral ischemia and that autophagy can be activated by antioxidants. The application of antioxidants or autophagy revulsive can reduce neuronal damage and significantly decrease the infarction area [145]. Thus, we can speculate that antioxidants might play a protective role in ischemic injury by inducing autophagy. There may

Table 1: The interplay between ROS and autophagy/mitophagy in neurological diseases.

Author	Year	Model (animal/cell)	Main idea	Effect of autophagy
Zhao et al. [144]	2016	MCAO/SD rats	FA deficiency simultaneously enhanced the activity of autophagy and induced the generation of oxidative stress following the MCAO model; oxidative injury seems to be involved in excessive activation of autophagy caused by FA deficiency.	Detrimental
Wenjing et al. [145]	2013	Mouse & neural cells	Autophagy is upregulated, and the level of ROS is elevated in the central nervous system after ischemia-reperfusion; Antioxidants can protect neural cells and decrease infarct volume possibly by activating the autophagic pathway of cells.	Protective
Kubota et al. [146]	2010	MCAO/SD rats	Chemical inhibitors of autophagy or lysosomes can delay the release of mitochondrial ROS to prolong the therapeutic time window. Ischemic insults will immediately initiate autophagy induction with undefined mechanisms, which significantly will impact ROS production and oxidative damage in vivo.	Detrimental
Dai et al. [147]	2017	OGD/cortical neurons	Sirt3 showed a protective role in eliminating intracellular H_2O_2, attenuating mitochondrial O_2^-, and promoting autophagy through the AMPK-mTOR pathway in neuronal ischemia.	Protective
Shao et al. [149]	2016	SH-SY5Y/neuronal cells	SIRT6-mediated autophagy contributes to oxidative stress-induced neuronal injury since inhibition of autophagy could prevent the detrimental effect of SIRT6 on cell survival, which could be attributed to attenuation of AKT signaling closely related to oxidative stress.	Detrimental
Khandelwal et al. [105]	2011	3xTg-AD mice	The autophagic removal of Aβ mediated by Parkin can attenuate oxidative stress and mitochondrial dysfunction to restore energy supply for a better modulation of autophagy in AD transgenic mice.	Protective
Giordano et al. [154]	2014	PD mouse model	Autophagy is proposed as an antioxidant protective pathway that can clear cumulative ROS and reverse established ROS-induced protein, DNA, and lipid damage independent of the disposal of radical scavengers.	Protective
Underwood et al. [155]	2010	Mouse cortical neurons	Autophagy can scavenge aggregate-prone proteins and increased ROS, while antioxidants can block autophagy and thereby counterbalance the benefits of autophagy and exacerbate neurodegeneration.	Protective
Dagda et al. [159]	2009	PD cell model/SH-SY5Y	Loss of PINK1 function can stir oxidative stress, which can then elicit coordinated autophagy and mitophagy for mitochondrial turnover by a removal of dysfunctional mitochondria.	Protective

be some more complicated mechanisms of crosstalk between autophagy and oxidative stress in need of further research. One study pointed to the finding that ischemic insults could immediately activate autophagy as a neuroprotective mechanism, which significantly affects ROS generation and oxidative toxicity. As well, pharmacological inhibition of autophagy or lysosomes can delay the mitochondrial ROS burst [146]. Scherz-Shouval and Elazar [15] have argued that ROS can upregulate autophagy through multiple signaling pathways. Sirt3 is a conserved deacetylase associated with biological functions such as energy metabolism, stress resistance, and mitochondrial redox homeostasis. Furthermore, it can positively regulate autophagy through the AMPK-mTOR pathway [147], which promotes neuronal survival within an in vitro oxygen and glucose (OGD) deprivation model of cerebral ischemia created by attenuating H_2O_2 and O_2^- [148]. Pharmacological or genetic inhibition of autophagy can ameliorate SIRT6-mediated neuronal injury, probably via attenuating AKT signaling closely related to oxidative stress in the OGD model of SH-SY5Y neurons [149]. Further, in vivo mechanistic studies are needed to verify the interplay of oxidative stress and autophagy. Furthermore, moderate activation of ROS can promote the translocation of Parkin to injured mitochondria and then incur Parkin-mediated mitophagy and ensure the integrity of mitochondria in ischemic brain injury [150] (Table 1).

4.2.2. Reactive Oxygen Species (ROS) and Autophagy (Mitophagy) in AD. Alzheimer's disease (AD) is one of the most common types of late-onset neurodegenerative diseases, hallmarked by a progressive loss of memory and cognition coupled with typical pathological features including neuritic plaques (NPs) and neurofibrillary tangles (NFTs) [46, 151]. Enhanced ROS and oxidative damage have been proven to be implicated in the evolution of neuronal dysfunction during the early events of AD [152].

Growing evidence suggests that spatial learning and memory deficits in AD may be tightly correlated with the impairment of the Nrf2-ARE pathway since Nrf2 knockout confers AD model mice with more sensitivity to neuronal

damage. Strikingly, some scholars have proposed that the interaction between oxidative stress and mitochondrial dysfunction may be involved in the process of AD because of the influence oxidative stress has on mitochondrial transport [153]. It has been observed that autophagic vacuoles with engulfed, defective mitochondria increased in the pyramidal neurons of AD patients [7]. The autophagic removal of damaged mitochondria and Aβ mediated by Parkin can attenuate oxidative stress and restore the energy supply so as to delay or prevent neurodegeneration in AD transgenic mice [105] (Table 1).

4.2.3. Reactive Oxygen Species (ROS) and Autophagy (Mitophagy) in PD. Parkinson's disease (PD) is a movement disorder with three outstanding clinical characteristics: bradykinesia, resting tremor, rigidity, and postural instability. Although the underlying etiology of PD is still far from clear, oxidative stress and mitophagy deficiency have been proposed as the principal elements in the development of dopaminergic neuronal death in the substantia nigra of PD patients.

Neurodegenerative diseases such as PD are often accompanied by increased oxidative brain damage coinciding with a reduction in antioxidants. This leads to dysfunctional mitochondria or protein aggregates that can be rescued to some extent by radical scavengers. Autophagy has been proposed as an endogenous, antioxidant, protective pathway that can clear accumulated ROS and reverse established ROS-induced protein, DNA, and lipid damage independent of the disposal of radical scavengers [154]. Protein accumulation and oxidative stress are pathologically pronounced in neurodegenerative diseases. Enhancing autophagy could scavenge aggregate-prone proteins and increased ROS, while antioxidants could block the benefits of autophagy and exacerbate neurodegeneration [155].

Mitochondria have a central role in redox regulation of autophagy as the generator and scavenger of ROS [156], but can be attacked when ROS exceed the scavenging activity. The dysfunction of mitochondria is a prominent initiating factor of nervous system diseases [157]. This dysfunction then amplifies oxidative damage, with the underlying assumption that the quality and quantity of mitochondria significantly affects neuronal function. Mitophagy was originally proposed to clear disturbed mitochondria after pathological stress in an attempt to restore homeostasis [158].

Dagda et al. discovered that knockdown of PINK1 in a recessive PD model can result in the accumulation of mitochondrial ROS, accompanied by clustered fragmented mitochondria and depolarized mitochondria which correlate with autophagy. More importantly, autophagy does play an essential role in limiting dopaminergic neuronal death in this genetic model and RNAi knockdown of genes necessary for inducing autophagy exacerbates the occurrence of PD [159] (Table 1).

Several studies have claimed that mitochondrial dysfunction and the existence of mitochondrial complex *I* defects also contribute immeasurably to the disease by playing a causative or consequential role in the exacerbation of oxidative stress in dopaminergic neurons. DJ-1, a causative protein of familial PD, is essential for modulating PINK/Parkin-mediated mitophagy [160]. Both DJ-1 and DJ-1-binding compounds have been identified as neuroprotective against oxidative stress in PD rats [161].

5. Conclusion and Perspective

Plenty of studies have repeatedly shown that ROS accumulation displays detrimental implications for the basic function and survival of neurons. ROS or oxidative stress can provoke autophagy, and autophagy can take part in the removal and repair of ROS-induced oxidative lesions through a variety of signaling pathways. But autophagic neuronal death will still result if cumulative ROS go beyond the scavenging activity of autophagy. At present, it appears to be contradictory that autophagy serves as a cellular self-purification mechanism, but hyperactivity or hypoactivity of autophagy is unfavorable for the normal functionality of neurons [162, 163]. After all, the predetermined threshold level of perfect autophagy is often blurred, particularly under a variety of disease courses. So, more relevant, constructive research should be undertaken without delay.

Mitochondria are thought to be crucial for neuronal function and fate by supplying energy and modulating redox status. It is well established that brain mitochondrial dysfunction or mitophagy defects are strongly associated with the initiation and progression of CI, AD, and PD. Neuronal mitochondrial impairments exhibit pronounced effects on mitochondrial membrane potential, leading to the prolonged opening of mPTP and an elevated production of ROS which can be rescued by mitophagy and ensuing mitochondrial turnover. Concurrently, treatment with mitochondria-targeted antioxidants substantially mitigates neuronal mitochondrial disturbance and oxidative damage [164, 165].

In summary, we provided a basic knowledge of ROS and autophagy/mitophagy and then expatiated specifically on the interrelation between ROS and autophagy as well as on their molecular regulatory mechanisms. Finally, we discussed the interplay of ROS and autophagy in CI, AD, and PD. Nonetheless, a lot of current work only focuses on the close interplay between ROS and autophagy/mitophagy in CI and PD, while there are few studies on how they are involved in AD and the underlying, precise, regulatory mechanisms are not well investigated. In the future, more basic research is needed to further excavate the correlation between autophagy/mitophagy and ROS together with their possible mechanisms in neurological disorders. Such research will lay a good foundation for pinpointing late-model drug targets and exploring aggressive therapeutic tactics that are applicable for the clinical treatment of such life-threatening neurological diseases.

Conflicts of Interest

The authors have no conflicts of interest.

Authors' Contributions

Congcong Fang and Lijuan Gu equally contributed to this work.

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Review Article

Multifaceted Roles of GSK-3 in Cancer and Autophagy-Related Diseases

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GSK-3 is a ubiquitously expressed serine/threonine kinase existing as GSK-3α and GSK-3β isoforms, both active under basal conditions and inactivated upon phosphorylation by different upstream kinases. Initially discovered as a regulator of glycogen synthesis, GSK-3 is also involved in several signaling pathways controlling many different key functions. Here, we discuss recent advances regarding (i) GSK-3 structure, function, regulation, and involvement in several cancers, including hepatocarcinoma, cholangiocarcinoma, breast cancer, prostate cancer, leukemia, and melanoma (active GSK-3 has been shown to induce apoptosis in some cases or inhibit apoptosis in other cases and to induce cancer progression or inhibit tumor cell proliferation, suggesting that different GSK-3 modulators may address different specific targets); (ii) GSK-3 involvement in autophagy modulation, reviewing signaling pathways involved in neurodegenerative and liver diseases; (iii) GSK-3 role in oxidative stress and autophagic cell death, focusing on liver injury; (iv) GSK-3 as a possible therapeutic target of natural substances and synthetic inhibitors in many diseases; and (v) GSK-3 role as modulator of mammalian aging, related to metabolic alterations characterizing senescent cells and age-related diseases. Studies summarized here underline the GSK-3 multifaceted role and indicate such kinase as a molecular target in different pathologies, including diseases associated with autophagy dysregulation.

1. GSK-3 Structure and Regulation

GSK-3 is a serine/threonine kinase existing as two isoforms named GSK-3α (51 KDa) and GSK-3β (47 KDa), expressed in most tissues and encoded by two different genes. According to the bodymap analysis available at IST Online Medisapiens (http://ist.medisapiens.com/#bodymap), the expression is ubiquitous but shows relevant differences in different tissues. For instance, GSK-3α is much less expressed in the nerves, ovary, and skin, while it is expressed at higher levels in the reticulocytes, appendix, whole blood, and pituitary gland. On the other hand, GSK-3β is much less expressed in the reticulocytes, dura mater, lymph node, and pancreas, while it appears expressed at higher levels in the blood NK cells and bone marrow granulocytes.

An alternative splice variant of GSK-3β, named GSK-3β2, has also been reported [1]. Both isoforms are monomeric and comprise a highly conserved catalytic domain (about 98% identity). Such high rate of similarity explains why the two isoforms phosphorylate the same targets [2]. The GSK-3 three-dimensional structure resembles that of mitogen-activated protein kinase (MAPK) family members and the fully active conformation depends on its interaction with the substrate which previously undergone a "priming phosphorylation event" by other kinases [3]. The larger mass of GSK-3α compared to GSK-3β is due to its

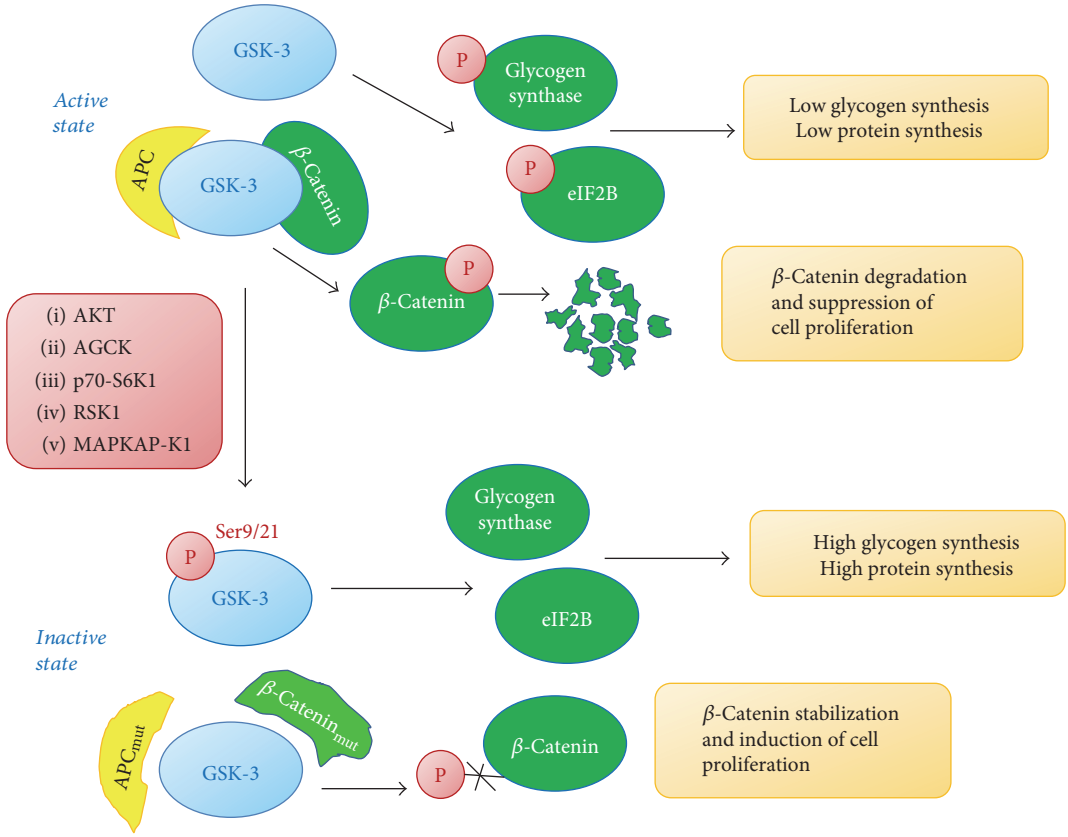

Figure 1: GSK-3 regulation.

glycine-rich N-terminal tail, responsible for the GSK-3α cytoplasm localization, while GSK-3β, which lacks the glycine-rich domain, has a nuclear and cytoplasmatic localization [4, 5]. Other main differences fall in their C-termini, showing only 36% identity in the last 76 residues of the two isoforms. Under basal conditions, both proteins are active. GSK-3β constitutive activation seems to occur *via* phosphorylation in tyrosine 216 [6]. Phosphorylation in N-terminal serine 21 and serine 9, respectively, of GSK-3α and GSK-3β by AKT leads to their inactivation and consequently glycogen and protein synthesis increase. The serine residue on GSK-3 has been also shown to be phosphorylated by other kinases, such as AGC kinases, p70 ribosomal S6 kinase-1 (p70-S6 K1), p90 ribosomal S6 kinase (RSK1), and MAPK-activated protein kinase-1 (MAPKAP-K1, also known as RSK). In addition to its posttranslational regulation through phosphorylation, GSK-3 activity may be modulated through its association with other proteins. In particular, GSK-3 interaction with axin has been well studied and demonstrated to be crucial for GSK-3-dependent regulation of canonical WNT signaling pathway [6]. A schematic representation of GSK-3 inhibition through phosphorylation by different kinases is shown in Figure 1.

2. Signaling Pathways Regulated by GSK-3

GSK-3 was originally demonstrated to play an important role in regulating glycogen synthesis, as one of the molecular events involved in insulin signaling. Insulin activates phosphatidyl-inositide 3-kinase (PI3K) which in turn activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), thus leading to AKT kinase phosphorylation. The latter phosphorylates and inhibits GSK-3, leading to dephosphorylation of GSK-3 substrates such as glycogen synthase and eukaryotic initiation factor 2B (eIF2B), finally promoting conversion of glycogen synthase to its active form and stimulating both glycogen and protein synthesis [3]. Amino acids have also been shown to inhibit GSK-3; this occurs *via* the mammalian target of rapamycin (mTOR) and the downstream S6K1 kinase [7]. Growth factors such as EGF may inhibit GSK-3 by both MAPK pathway and PI3-kinase/AKT pathway, and tumor-promoting phorbol esters can inhibit GSK-3 via MAPK cascade [8]. Furthermore, a WNT-induced inhibition of GSK-3 has been described. In the absence of WNTs, GSK-3 is active and phosphorylates axin, β-catenin, and adenomatous polyposis coli (APC). Under this condition, β-catenin undergoes ubiquitin-mediated proteolytic degradation. When WNTs bind their frizzled receptors, through the key transducer Dishevelled (DVL) phosphoprotein, stabilization and accumulation of β-catenin occur; this event is dependent on GSK-3 inhibition due to phosphorylation at a residue different from that targeted by AKT [3]. In fact, although AKT signaling leads to inhibition of GSK-3 via serine phosphorylation, AKT signaling does not cause stabilization and accumulation of β-catenin [9, 10]. It has also shown β-catenin accumulation in the presence of highly active GSK-3, and this is dependent on APC or β-catenin

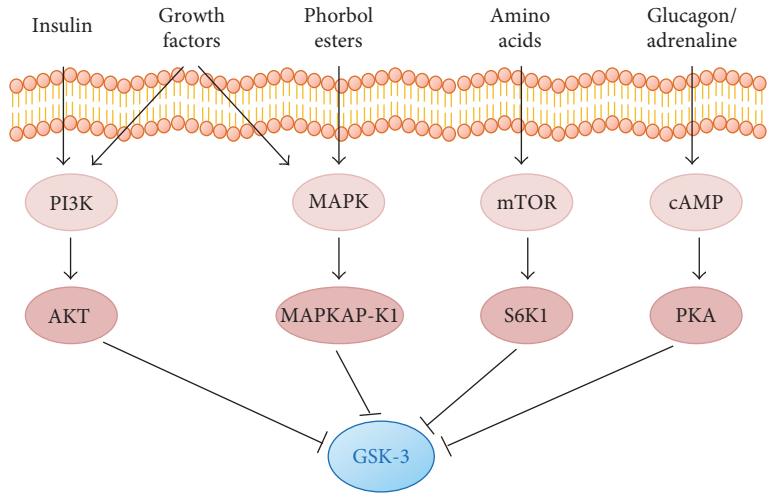

Figure 2: Signaling pathways leading to GSK-3 inactivation.

mutations [11, 12]. Given the role of active GSK-3 in promoting degradation of oncogenic proteins such as β-catenin, it may acquire tumor suppressor properties. Conversely, upon canonical WNT signaling, inactive GSK-3 fosters cell proliferation by β-catenin stabilization thus acquiring tumor-promoting activity (Figure 1). Since β-catenin is an essential component of cadherin-based adhesion junctions, GSK-3 also regulates cell adhesion via β-catenin accumulation. Interestingly, it has been also shown that WNT signaling does not directly inactivate GSK-3 but more likely disrupts the formation of the "β-catenin destruction complex" [13].

GSK-3 can be phosphorylated and inhibited by cyclic AMP- (cAMP-) dependent protein kinase/protein kinase A (PKA) in the presence of high cAMP levels, following glucagon or adrenaline stimulus. Remarkably, GSK-3 phosphorylation can also be achieved by incubation with cAMP-elevating agents or cAMP analogues [14]. A schematic representation of signaling pathways responsible for GSK-3 inhibition is shown in Figure 2. The inhibition of GSK-3 by the different pathways generally leads to dephosphorylation of its substrates. Phosphorylation of GSK-3 substrates generally leads to their inactivation, and many substrates require an additional "priming phosphorylation event" which is performed by a different kinase and occurs at a site located C-terminally to the site phosphorylated by GSK-3. Extensive lists of GSK-3 substrates or GSK-3 binding proteins have been reported and include amyloid precursor protein, APC, ATP-citrate lyase, axin, axil, β-catenin, c-jun, Jun B, Jun D, Ci155, C/EBP alpha, CRMP2, CRMP4, CREB, CTP, cyclin D1, dystrophin, eIF2B, glycogen synthase, glucocorticoid receptor, heat shock factor 1, hnRNP, K-casein, KRP, MAB 1B, MAP 2, MAP 2C, MITF, c-Myc, L-Myc, alpha NAC nascent polypeptide-associated complex, NCAM, NDRG1, NDRG2, neurofilament L, neurofilament M, neurofilament H, Notch 1C, p21 CIP1, p53, presenilin, pyruvate DH, PP1 G-subunit, protein phosphatase inhibitor 2, stathmin, synphilin-1, RSK1, and Tau (https://thebiogrid.org/ and http://www.genecards.org/).

GSK-3-dependent substrate phosphorylation may represent a signal toward their degradation. In fact, when GSK-3 phosphorylates cyclin D1 at threonine 286 and c-myc at threonine 58 they undergo ubiquitylation and proteolytic degradation. For such a reason, upon GSK-3 inhibition, growth factors may lead to both cyclin D1 and c-myc stabilization. Also, the transcription factor c-jun may be phosphorylated by GSK-3 and this event suppresses its DNA binding activity. Therefore, GSK-3 inhibition is able to enhance c-jun potential to stimulate the transcription of several genes including those involved in cell cycle progression [15].

3. Role of GSK-3 in Apoptosis

It is now clear that GSK-3 plays a pivotal role in numerous cellular functions, other than regulator of glycogen metabolism. As reported below, active GSK-3 has been shown to induce apoptosis in some cases and to inhibit apoptosis in other cases. Cooper and collaborators first demonstrated that, while GSK-3 overexpression induces apoptosis in different cell lines (i.e., pheochromocytoma PC12 cells and Rat-1 fibroblasts), overexpression of a GSK-3 inactive mutant prevents apoptosis [16]. Other studies performed using specific GSK-3 inhibitors confirm this finding [17]. We have previously addressed this issue in skeletal muscle tissue and demonstrated that decreased GSK-3β serine-9 phosphorylation leads to increased active caspase-3 and cytochrome *c* release [18]. GSK-3β has been shown to be directly involved in cell death mediated by PI3K/mTOR inhibitor and by pan-histone deacetylase (HDAC) inhibitor, in lymphoma cell lines [19]. Interestingly, trichostatin A, a histone deacetylase inhibitor (HDACI), induces apoptosis through GSK-3β in MCF-7 breast cancer cells [20], and a specific GSK-3 inhibitor (SB-415286) induces apoptosis in different leukemia cell lines [21]. In neurons, GSK-3β exerts a proapoptotic action inducing mitochondrial translocation of the proapoptotic Bcl-2 family member Bax, which occurs after GSK-3β-dependent phosphorylation of Bax in Ser163 [22]. Moreover, GSK-3β inhibition significantly reduces hepatic apoptotic cell death in response to D-galactosamine/LPS-induced liver injury [23] and improves the survival of mice with polymicrobial sepsis, ameliorating liver injury, with a mechanism

involving its ability to inhibit inflammatory response by modulation of NF-?B and CREB activation [24]. These data suggest that inhibition of GSK-3β may act as a relevant complementary strategy to the antibiotic treatment opening an interesting scenario in the development of novel antimicrobial strategies. As a further indication of the role of GSK-3 in apoptosis regulation, GSK-3β KO mouse has been reported to die in utero and this phenotype is likely dependent on an apoptosis defect [25]. As discussed in more details in the next section, GSK-3 may have a relevant effect on cancer cell apoptosis, likely via β-catenin. In fact, on one hand, it has been demonstrated that GSK-3 regulates axins, intracellular β-catenin antagonists, and cell fate regulators, while on the other hand inhibition of GSK-3 enhances TRAIL-induced apoptosis [26] as well as sorafenib-induced apoptosis in melanoma cells [27].

4. Opposite Role of GSK-3 in Cancer Progression/Setup

GSK-3 role in cancer progression is largely investigated and still debated. In fact, in some cases, GSK-3 activity has been associated with tumor progression, while in other cases suppression of GSK-3 activity by different kinases has been associated with cancer progression, for instance, by stabilizing components of the β-catenin complex. GSK-3β inhibition leads to β-catenin activation and tumor cell proliferation [28]. However, GSK-3 is overexpressed in various cancer conditions such as colon, liver, ovarian, and pancreatic tumors and GSK-3β downregulation inhibits pancreatic cancer growth, angiogenesis, and vascular endothelial growth factor expression [29–32]. GSK-3 role in cancer is often dependent on GSK-3-driven mammalian target of rapamycin (mTOR), a signaling molecule crucial in cell proliferation. mTOR is found in two complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). Signaling through mTORC1 is involved in tumor progression, and remarkably, GSK-3 inhibitors have been shown to inhibit mTORC1 activity [33]. Recently, GSK-3 involvement has been demonstrated in a study reporting that differentiation-inducing factor-1 displays a strong antimelanoma activity exerted in two ways, the first (i.e., antiproliferation action) involving a GSK-3-dependent degradation of cyclin D1 and c-Myc and the second (i.e., antimigration and anti-invasion) involving a GSK-3-independent mechanism [34]. Further, GSK-3 directly induces growth and survival in human melanoma cells, by increasing levels of the Pax3 transcription factor [35].

According to GEO database (https://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1375), we observed that 63 samples of human melanoma and benign nevi reported in the dataset GDS 1375 [36] show GSK-3α expression significantly upregulated in melanoma biopsies as compared to benign nevi human biopsies (1200 units versus 901 units, $p < 0.0001$), while GSK-3β expression appears unmodified (791 units versus 680 units, $p < 0.2$). Such observation was confirmed by the additional data reported in IST Online Medisapiens dataset (http://ist.medisapiens.com) collected from the 355 samples of melanoma and healthy skin, and all together support the hypothesis of a differential role of GSK-3α and GSK-3β in melanoma biology.

β-Catenin regulation by GSK-3β has been shown to play a key role in hepatocellular carcinoma (HCC). An enhanced activation of WNT/β-catenin pathway is often found in several types of cancers; it may be considered an early event in hepatocarcinogenesis and correlates with an aggressive phenotype [37]. In addition, the liver carcinogenesis induced by HCV has been related to the HCV core protein ability to stabilize β-catenin by inhibiting GSK-3β [38]. Furthermore, in HCC, several molecular mechanisms involving genetic and epigenetic alterations have been shown [39]. One mechanism involves insulin and IGF-1. They inhibit GSK-3β [40], leading to nuclear localization of β-catenin [41] which binds its nuclear targets, such as TCF/LEF-1, and induces gene transactivation and tumor formation [42].

Usually, AKT is activated in human cancers, including carcinomas, glioblastoma multiforme, and various hematological malignancies. Noteworthy, while activated AKT inhibits GSK-3 through the phosphorylation of GSK-3 at Ser21/Ser9, however, such inactivation does not always affect β-catenin levels in the cell and does not completely inhibit GSK-3. For instance, two pancreatic cancer cell lines, PANC1 and ASPC1, exhibit amplification of AKT and high levels of AKT RNA and protein [43] but also highly active GSK-3β suggesting that, although some pools of GSK-3 can be phosphorylated by AKT at Ser21/Ser9 and inhibited, other pools of GSK-3 may remain active in cancer cells [31]. Moreover, another study has shown high levels of active AKT in human colorectal carcinomas, but levels of inactive phospho-GSK-3β Ser9 are lower than in their normal counterparts [30]. Altogether, these studies suggest that AKT activation and GSK-3 inhibitory phosphorylation are not always correlated *in vivo* in human tumors and part of GSK-3 remains active in cancer cells irrespective of AKT activation.

Data available indicate GSK-3β as a crucial gatekeeper to maintain a regular cell proliferation rate and conditions favorable to cell death activation. This suggests that the persistent inhibition of GSK-3β may favor oncogenic conditions. The autocrine stimulation of an IGF-1 R-dependent signaling pathway is one of these conditions. Moreover, GSK-3 interacts with other signaling pathways implicated in HCC pathogenesis, such as Notch, Hedgehog (HH), and TGF-β pathways. Many studies demonstrate the aberrant activation of HH [44] and Notch signaling [45]. In the latter, GSK-3 is an important component, although its role remains controversial. In fact, in some studies, GSK-3 activity has been reported to enhance nuclear localization and transcriptional activity by phosphorylation of two domains in Notch1 intracellular portion [46]. On the other hand, other studies report that GSK-3 phosphorylates and decreases Notch protein levels and downregulates its transcriptional activity [47]. Finally, the TGF-β pathway may have dichotomous function, with both pro- and antitumor activities. In fact, in early steps of hepatocarcinogenesis, TGF-β shows tumor-suppressive properties while in late stage, it promotes tumor progression by stimulating epithelial-mesenchymal transition (EMT), cell invasion, and cancer metastasis [48]. In hepatocytes, TGF-β, through a Src-dependent pathway, activates ERK5 that can

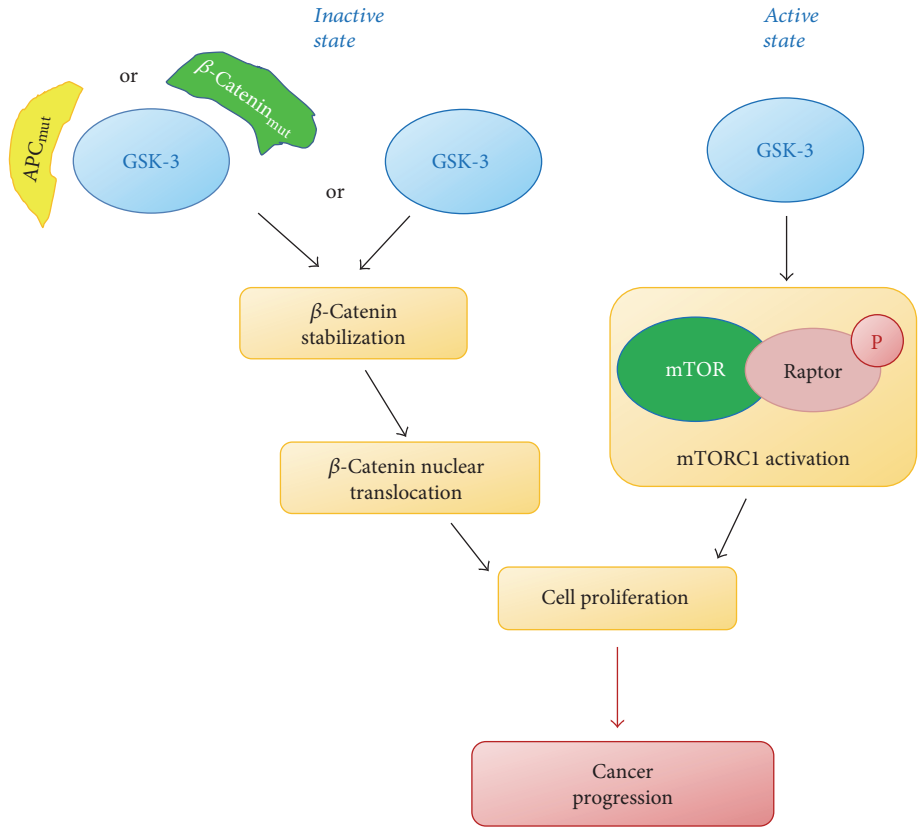

Figure 3: Opposite role of GSK-3 in cancer.

phosphorylate GSK-3β on serine 9, inhibiting its activity [49]. TGF-β, by inhibiting GSK-3 kinase activity, interferes with phosphorylation of the tumor suppressor hepatocyte nuclear factor 4 alpha (HNF4α), a transcription factor controlling the expression of EMT master genes such as SNAI1; this results in its functional inactivation and contributes to EMT progression.

Cholangiocarcinoma (CCA) is the second most common primary hepatobiliary cancer that originates from biliary epithelium cells known as cholangiocytes [50, 51]. GSK-3β plays an important role in CCA, by mediating the cross-talk of PI3K/AKT and WNT/β-catenin pathways directly controlling cell growth in a cholangiocarcinoma setup [52]. Remarkably, GSK-3 α/β phosphorylation in serine 21/9 appears to be strongly increased in cholangiocarcinoma tissues as compared to normal biliary tissues and to be significantly associated with tumor progression. Also, P-glycoprotein (P-gp) is intrinsically overexpressed in many tumors, affecting the colon, rectum, pancreas, liver, kidneys, and bile ducts [53]. It is known to play a pivotal role in multidrug resistance (MDR), which reduces chemotherapy efficacy in CCA [54]. For that reason, several potent P-gp-dependent MDR reversers have been studied and the saponin mixture β-escin combined with other drugs such as 5-FU and VCR has shown remarkable inhibitory and synergic effects in CCA cells [55]. Interestingly, β-escin increases cholangiocarcinoma cells line sensitivity to chemotherapy, by inducing GSK-3β phosphorylation and dephosphorylation at tyrosine-216 and serine-9, respectively, leading to β-catenin degradation [55]. Finally, prostaglandin E2 (PGE2) is known to induce cholangiocarcinoma cell proliferation and invasion in a GSK-3-mediated way [56]. Altogether, all these studies reveal that, although its protumor or antitumor role is still debated depending on the cellular context, GSK-3 may be considered a promising molecular target in different tumors. A schematic representation illustrating the opposite models of GSK-3 involvement in cancer is shown in Figure 3. It suggests that different GSK-3 modulators (activators or inhibitors) should be further explored to address their specific effect in cancer treatment.

5. Role of GSK3 in Autophagy

Autophagy is a complex molecular mechanism involved in disassembling unnecessary or dysfunctional cellular components through double-membrane vesicles named autophagosomes, ultimately fusing with lysosomes, leading to their degradation through lysosome hydrolases. This process is usually activated under nutrient deprivation [57]. Autophagy starts with the formation of an isolation membrane called phagophore; then, the phagophore edges fuse to form a double-membrane vesicle, named autophagosome, sequestering the cytoplasmatic material to be eliminated. This process is performed through a complex molecular machinery including mTOR, which therefore represents a critical autophagy regulator [58, 59]. mTOR kinase is a sensor of intracellular amino acids, ATP, and hormones and acts as an autophagy inhibitor. It is inhibited by the autophagy inducer rapamycin, controls the autophagy onset, and is responsible

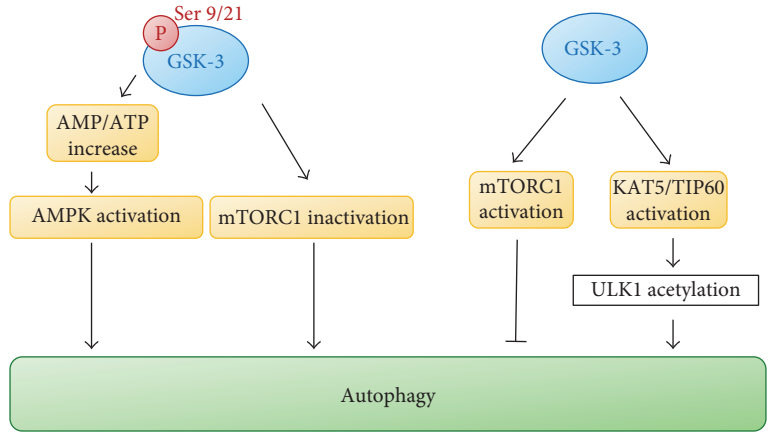

Figure 4: Models of GSK-3 involvement in autophagy.

for S6K and 4EBP1 phosphorylation [60]. Two ubiquitin-like conjugation pathways are involved in autophagosome formation, namely, the autophagy-related (ATG) 8 and ATG12 protein systems. Such two systems control phosphatidylethanolamine conjugation to mammalian LC3. As a result, the soluble LC3-I is converted to LC3-II, recruited to the autophagosomal membrane; therefore, such molecule is usually exploited to monitor autophagy [61]. Despite the large investigation regarding the role of autophagy in tumor formation and metabolism, its precise function is still debated since it has demonstrated both tumor-promoting and tumor-suppressing properties [62]. Autophagy, by releasing metabolic precursors necessary for macromolecular biosynthesis or ATP generation, makes energy available to tumor cells undergoing metabolic stress. On the other hand, autophagy genes are frequently monoallelically deleted, silenced, or mutated in different human tumors, thus supporting the autophagy tumor-suppressing properties [63]. Therefore, while during cancer initiation, autophagy may suppress tumor progression and autophagy deregulation may contribute to genomic instability; in the later stages, it may facilitate tumor progression supporting cancer cell survival, particularly in the presence of therapy-induced stress. GSK-3 role in autophagy regulation has been studied in the past few years. GSK-3 inhibits autophagy through the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). In fact, overexpression of either GSK-3α or GSK-3β activates mTORC1 and suppresses autophagy in MCF-7 breast cancer cells. Conversely, treating cells with GSK-3 inhibitors inhibits mTORC1 activity and increases autophagic flux [33]. It has been clarified that GSK-3 regulates mTORC1 by phosphorylating the mTOR-associated scaffold protein raptor (regulatory-associated protein of mTOR) on Serine 859. GSK-3 inhibition reduces mTOR and raptor interaction leading to reduced phosphorylation of both p70S6K1 and ULK-1 and to increased autophagic flux [64]. In human breast cancer cells, GSK-3 overexpression increases the autophagosome number by autophagic flux inhibition. This activity has been directly related to reduced lysosomal acidification triggered by GSK-3 [33]. Furthermore, GSK-3 inhibition induces prosurvival autophagy in human pancreatic cancer cells. This occurs through GSK-3 dependent regulation of the transcription factor EB (TFEB), that is, a master regulator of autophagy and lysosomal biogenesis [65]. In a prostate cancer cell model, inhibition of GSK-3β activity leads to a significant increase of AMP/ATP ratio, a strong trigger of AMPK activation, thus leading to autophagy induction [66]. Inoki and colleagues have also shown that GSK-3 inhibits mTOR pathway by phosphorylating the tumor suppressor TSC2 in an AMPK-priming phosphorylation-dependent manner. Therefore, sequential phosphorylation of TSC2 by AMPK and GSK-3 occurs and these events may lead to mTOR pathway inhibition [67]. GSK-3 commonly accepted involvement in autophagy regulation is schematically represented in Figure 4.

Alterations of autophagic pathways have been extensively investigated in degenerative diseases and have been shown to be the central mechanisms in the pathogenesis of amyotrophic lateral sclerosis. Interestingly, a small heterocyclic GSK-3 inhibitor is able to induce the recovery of neurological symptoms in amyotrophic lateral sclerosis condition [68]. Autophagy impairment has been reported in other neurodegenerative processes. In fact, upon neurotoxin intoxication, astrocytes undergo autophagic flux block that can be rescued by rapamycin or by GSK-3β inhibition [69]. GSK3 overactivity has been reported to occur in sporadic Alzheimer's disease (AD) cases and therefore may play an important role in disease progression. GSK-3 mediates the hyperphosphorylation of tau (one of the brain microtubule-associated proteins), the increased production of β-amyloid from β-amyloid precursor protein (via β and γ secretase-mediated cleavage), and ultimately leads to autophagy impairment. More in detail, GSK-3α, but not GSK-3β, has been shown to regulate β-amyloid precursor protein cleavage resulting in the increased production of β-amyloid plaques. Since the discovery of its involvement in AD [70], GSK-3 has been proposed as a new target enzyme and is expected to provide a novel avenue for therapeutic intervention in AD. In Parkinson's disease (PD), the GSK3-β inhibitor lithium decreases the aggregation and phosphorylation of α-synuclein and leads to increased autophagy. Conversely, GSK3β activation depresses autophagy and increases the total protein level and phosphorylation of α-synuclein [71].

Data reported in literature indicate that autophagy is regulated by GSK-3 mostly via mTORC1. It has also been clarified that GSK-3, in the absence of growth factors, is able to activate the acetyltransferase KAT5/TIP60, which in turn stimulates the protein kinase ULK1 to induce autophagy [72]. Remarkably, GSK-3 seems to play a key role also in stemness; in fact, inhibition of both GSK-3 and mTORC1 induces a proautophagic gene signature in hematopoietic stem cells, which is crucial to maintain their self-renewal ability [73].

In the liver, several autophagy pathways have been identified and characterized [74]. Selective autophagy contributes to several physiological functions, representing a mechanism exploited by hepatocytes in order to modulate their metabolic capability [74]. Hepatic autophagy mostly depends on the fasting–feeding cycle and is under hormones and amino acid control [74]. Hepatic autophagy has a key role in the adaptation to starvation, inducing glycogenolysis, lipolysis, and protein catabolism. Furthermore, quality and quantity control of mitochondria and peroxisomes can directly regulate hepatic metabolism through β-oxidation [74]. In fasting, early-phase glucagon leads to GSK-3 inhibition and promotes hepatocyte glycogenolysis in order to maintain blood glucose levels [75]. Moreover, upon nutrient deprivation, hepatocytes upregulate the transcription of genes related to β-oxidation and autophagy, thus leading to lipophagy with subsequent β-oxidation and ketone body production [74, 76]. Of notice, differently from GSK-3β KO mice, GSK-3α KO mice are not embryonically lethal although they have metabolism defects such as enhanced glucose and insulin sensitivity [77] further supporting the involvement of GSK-3 in autophagy-dependent metabolic processes. Furthermore, it has been suggested that persistent phosphorylation of GSK-3β may have a fundamental impact on glycogen metabolism and cell growth in hepatoma cells [78].

Given its role in metabolic balance and organelle quality control, an unbalance or malfunction of autophagy pathways in hepatocytes has been associated with the pathogenesis of several liver diseases, including nonalcoholic fatty liver disease (NAFLD), alcoholic fatty liver (AFL), viral hepatitis, and liver cancer [79]. NAFLD is one of the most important causes of liver-related morbidity in obese children and adults [80–82]. Both NAFLD and AFL are characterized by hepatocyte steatosis. In NAFLD, fatty liver is mostly due to continuous dietary intake of excess dietary fat in the absence of excess alcohol consumption [79, 81, 82]. Differently, in AFL, steatosis is due to ethanol metabolism which leads to increased production of highly reactive acetaldehyde, fatty acid ethyl esters, and phosphatidylethanol [83]. Interestingly, both NAFLD and AFL are histologically characterized by impaired autophagy associated with prominent SQSTM1 protein accumulation in the form of cytoplasmic inclusions, histologically known as Mallory bodies [74, 84]. It has been suggested that inhibition of GSK-3β activity may be considered an important strategy to reverse the imbalanced oxidation and the impaired autophagy and ameliorate liver conditions [85].

Selective autophagy in hepatocytes may represent a defense mechanism against lipid accumulation [86]; however, lipotoxicity effects can prevail and suppress autophagic activity [87]. In fact, autophagy enhancement using pharmaceutical agents alleviates liver steatosis [88, 89] and contributes to Mallory body degradation [90]. NAFLD progression involves inflammation (nonalcoholic steatohepatitis (NASH)), fibrosis, and cirrhosis. In this context, the activation of hepatic stellate cells (HSCs) plays a key role in the progression toward fibrosis and cirrhosis [86]. Under normal conditions, HSCs are quiescent vitamin Astoring cells; however, in a diseased liver, HSCs are activated and change to myofibroblast-like cells. Activated HSCs acquire proliferative, contractile, and inflammatory properties and produce extracellular matrix compounds, thus resulting in fibrogenesis [91]. Interestingly, during this process, quiescent HSCs lose their lipid stores and autophagy may act by cleaving retinyl esters within cytoplasmic droplets [91]. It should be noted that selective knockout of autophagy-related genes in mouse HSCs inhibits experimental induced fibrogenesis [79, 92]. Thus, autophagy may support HSC activation resulting in enhanced fibrogenesis [91]. Therefore, although autophagy may have beneficial effect on hepatocyte steatosis in NAFLD, it may also induce HSC activation resulting in enhanced fibrogenesis [74].

Besides the role in hepatocytes and HSC, autophagy pathways are also investigated in the pathogenesis of biliary tree disorders [93]. Fibrosing cholangiopathies are a heterogeneous group of diseases affecting cholangiocytes (i.e., the parenchyma cells lining bile ducts) and comprising primary biliary cholangitis (PBC), primary sclerosing cholangitis, and biliary atresia [94]. Accumulation of LC3-positive vesicles and p62 aggregation were described in primary biliary cholangitis; autophagy deregulation may induce cholangiocyte senescence, which in turn is involved in the immune-mediated bile duct pathologies. Cholangiocytes can acquire a senescence-associated phenotype responsible for aberrant expression of chemokines, cytokines, and growth factors that can interact with pathogen-associated molecular pattern. Moreover, since mitochondria represent a major target of autophagy, deregulated mitochondria autophagy may be involved in the autoimmune pathogenesis occurring in PBC [95]. Finally, in primary sclerosing cholangitis, autophagy and senescence have been associated with the occurrence of epithelial to mesenchymal transition traits in cholangiocytes and biliary tree stem cells, with dysplasia features [96]. Interestingly in the last years, a novel mechanism implicating GSK-3β in TGF-β-induced EMT program has been reported [97]. Given the role of GSK-3 in regulating autophagy and its role in promoting metabolic changes toward the anabolism, GSK-3 may be considered as a potential target to counteract liver injury associated with autophagy impairment and senescence processes also in biliary tree disorders.

6. Role of GSK-3 in Oxidative Stress and Autophagic Cell Death

Oxidative stress occurs when the balance between reactive oxygen species (ROS) production and elimination is altered leading to accumulation of ROS which profoundly affects lipids, proteins, and DNA. Mitochondria are both great

producers and main targets of ROS; therefore, they play a central role in oxidative homeostasis. As a consequence of oxidative damage, mitochondrial permeability transition (MPT), a nonselective permeabilization of mitochondria inner membrane, may occur, usually followed by necrotic or apoptotic cell death [98]. GSK-3 activity is induced by ROS and it is involved in MPT. More in detail, GSK-3 is able to direct MPT through the phosphorylation of different targets and GSK-3 inhibition is known to protect from MPT [99]. In addition, GSK-3β inhibition has been shown to be required for the stability of Nrf2 transcription factor, a key regulator of the cellular defense against oxidative stress [100].

The prooxidative involvement of GSK-3 overexpression may explain at least in part its role in the pathogenesis of several disorders including cancer [99] as well as many neurological disorders including bipolar disorder [101] and AD [102].

Oxidative stress is often associated with different types of liver injury and plays an important role in the mechanism of acute liver failure (ALF) [103]. The effects of oxidative stress are balanced by antioxidant activities with a variety of enzyme and nonenzyme-mediated mechanisms. Active oxygen-scavenging systems include enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase, while nonenzymatic antioxidants include GSH, vitamin C, and vitamin E [104]. SOD and GSH activity in ALF is significantly lower as compared to normal controls. The oxidation status enhances paralleling ALF progression, whereas the antioxidants are reduced, resulting in a severe oxidative stress in ALF and in the progression of liver injury [85]. Oxidative stress regulates hepatocyte injury and death, and GSK-3β appears to be critical for their regulation in ALF. GSK-3β activity is depressed at an early stage of ALF and then goes back to high levels in the advanced ALF, further suggesting that GSK-3β may have a role in ALF progression. In hepatic ischemia/reperfusion (I/R) injury, that is, the most common cause of acute hepatic failure (after liver transplantation, hepatectomy, trauma, and shock), reperfusion following prolonged ischemia is related to the mitochondrial dysfunction, which induces liver apoptosis [105]. The impairment of oxidative phosphorylation and induction of MPT are critical determinants for such mitochondrial dysfunction [106] and are dependent on GSK-3β activity [107]. It has also been demonstrated that propofol, a drug used to induce and maintain anesthesia, may protect several tissues from I/R injury [108] supporting their mitochondrial function, thanks to GSK-3β inhibition which restrains MPT, preventing the cytochrome C release, mitochondrial swell, and mitochondrial membrane potential collapse [105].

GSK-3 has been also reported to play a role in regulating autophagic cell death. Under such condition, extensive autophagy does not provide cytoprotection but triggers cell death. Overexpression of Aurora-A kinase, a serine/threonine protein kinase, enhances mTORC1 activity by antagonizing GSK-3β activity, thus conferring resistance to autophagic cell death [109]. Furthermore, in a model of mesangial cells, cadmium has been demonstrated to be able to induce autophagic cell death through a GSK-3-regulated signal-transduction pathway. Serine 9 phosphorylation (i.e., the phosphorylation leading to the GSK-3β inhibition) decreases after cadmium treatment and, in turn, a specific GSK-3β inhibitor decreases cadmium-induced autophagic cell death. Remarkably, GSK-3 activation after cadmium treatment is a consequence of ROS elevation and a ROS scavenger is able to counteract autophagic cell death [110, 111]. Conversely, activation of AKT and GSK-3β inhibition suppresses cytodestructive autophagy in hippocampal neurons [112]. Furthermore, in neural stem cells, following insulin withdrawal, both pharmacological and genetic inactivation of GSK-3β significantly decreases autophagic cell death [113]. In addition, GSK-3β-mediated phosphorylation of BCL2 family member MCL1 has been demonstrated to induce axonal autophagy and axonal degeneration [114]. Altogether, these data indicate that besides controlling oxidative stress cellular response, GSK-3 may be also involved in prodeath autophagy.

7. GSK-3β-Specific Inhibitors: Using GSK-3 as a Pharmacological Target

Modulation of GSK-3 activity via pharmacological intervention may represent a valuable strategy to control autophagy and other conditions. In fact, GSK-3 is emerging as a possible therapeutic target for many diseases, and selective GSK-3 inhibitors are now available. Numerous studies show that GSK3 action supports cancer cells and suggest that its inhibition may have therapeutic benefits. However, as highlighted above, GSK-3 role in tumor development is still controversial. Many GSK-3 inhibitors have been developed and may have an application in GSK-3 overexpressing tumors [115]. The cation lithium is the first inhibitor to be discovered. Other metal anions such as copper, beryllium, mercury, and zinc have also been shown to interfere with GSK-3 activity. Other known GSK-3 inhibitors are chemical compounds including natural substances as well as synthetic ATP-competitive inhibitors, non-ATP-competitive inhibitors, and substrate-competitive inhibitors [66, 116]. An issue regarding ATP-competitive inhibitors may concern their lack of specificity; namely, they interfere with the phosphorylation of many substrates, giving potential oncogenic effects [3]. Some GSK-3 inhibitors have been used in clinical trials and are well-tolerated in cancer patients [117]. Significant clinical improvements have been shown in cutaneous T-cell lymphoma patients treated with valproate, which inhibits either GSK-3 isoforms [118].

Different therapeutic strategies to treat leukemia have been shown to involve mechanisms leading to GSK-3 activation often by suppressing PI3K/AKT pathway. For instance, a specific AKT inhibitor induces apoptosis in T-cell acute lymphoblastic leukemia (ALL) through a mechanism partially dependent on GSK-3 activation [119]. A GSK-3 inhibitor named PDA-66 shows some promise in preclinical studies using ALL cells [120] while GS-87, a highly specific inhibitor of GSK3, has been shown to induce differentiation of AML cells [121]. Nevertheless, the potential differentiating effect of GSK3 inhibitors needs to be further explored. The selective GSK-3α and GSK-3β inhibitor LY2090314 shows very high cytotoxic activity in melanoma cells, both resistant and nonresistant to BRAF inhibitor. Such activity was strongly

associated with β-catenin stabilization. *In vivo* confirmation of such data further support the potential efficacy of GSK-3 inhibitors in melanoma [122].

While different GSK-3 inhibitors have been evaluated in several pathologies and are well-tolerated in leukemia and pancreatic cancer patients, no clinical trials have been performed or are currently ongoing in HCC patients. Only preclinical studies are available on GSK-3 inhibitors in HCC. Indeed, developing novel GSK-3 inhibitors might be crucial to identify novel GSK-3 substrates and novel GSK-3 functions specific for one of the two isoforms. GSK-3 inhibitors have been tested in neurodegenerative conditions [123]. Unfortunately, sodium valproate [124] and tideglusib (a non-ATP competitive GSK-3 inhibitor) have both shown no significant effect in progressive supranuclear palsy [125, 126] while contrasting results are raised for tideglusib-treated Alzheimer's disease patients in two different clinical trials [127, 128]. Nevertheless, significant clinical improvements have been shown in valproate-treated patients affected by chronic migraine [129].

8. Does GSK-3 Counteract Mammalian Aging?

The GSK-3 ability to regulate numerous cellular processes through a number of signaling pathways important for cell proliferation, stem cell renewal, apoptosis, and development is widely accepted [130]. Because of its multifunctional role, GSK-3 strongly affects the first stages of human diseases as well as regulates age-related pathologies. Four main theories underlying aging molecular process are now generally accepted. Three of them are based on telomere loss, somatic mutation, and mitochondrial action. These hypotheses take into account, respectively, telomere shortening dysfunction, forms of DNA damage exciding DNA repair capacity, and mutation of mitochondrial DNA impairing ATP production. The fourth theory regards the waste accumulation, that is, it hypothesizes the aging results from toxic protein accumulation and alteration of degradative mechanisms such as lysosome-mediated autophagy [131, 132].

Metabolic alterations such as mitochondrial dysfunction, as previously mentioned, characterize senescent cells displaying structural features such as enlarged volume, increased granularity, and oxidative stress, all falling under GSK-3 control. Kim and colleagues [133] demonstrated that different anabolic processes, such as lipogenesis, glycogenesis, and protein synthesis increase during senescence in primary cell cultures. Consequently, the mass of senescent cells is augmented. Such increase is accompanied by ROS overproduction caused by defective respiration [134]. Oxidative stress induces and maintains the senescence cellular phenotypes since mitochondrial DNA is susceptible to oxidative damage. GSK-3 inactivation through phosphorylation plays a key role in these aging processes; in fact, GSK-3 is directly involved in glycogen accumulation as well as in protein synthesis activation, characterizing senescent cells [135]. The correlation between mitochondrial defects and metabolic changes related to age as well as the link with GSK-3 has been demonstrated by Kim and colleagues [133] using immortalized human liver cell, Chang cells, exposed to deferoxamine to induce senescence. Deferoxamine augmented GSK-3 phosphorylation at both serine 9 of GSK-3β and serine 21 of GSK-3α causing strong glycogen accumulation. Remarkably, the increase of the intracellular organelles like lysosomes and mitochondria [136], endoplasmic reticulum, and Golgi as well as total cell lipid content, represents a defense response to oxidative stress and a senescence factor. Namely, SREBP1 transcription factor expression, the major modulator of lipogenic enzyme modulator [137], is a GSK-3 target and increases in cell senescent systems. According to this finding, Kim and colleagues [133] observed that GSK-3 inhibition augmented cellular lipogenesis and membranous organelle mass. Grune and colleagues described an increase in the nonmembranous organelles [138]. This effect is related to a higher cellular anabolism during senescence, when cells are exposed to persistent oxidative stress with potential damage of cellular organelles.

Hence, GSK-3 inhibition leads to intracellular ROS overproduction thus stimulating mitochondrial damage. Furthermore, GSK-3 controls master factors in anabolic activation (namely, eIF2B, glycogen synthase, and SREBP1). Therefore, GSK-3 can be considered a main factor of the metabolic changes towards the anabolism shift observed in senescence.

Interestingly, some medicinal plants display antiaging effects shown to be linked to GSK-3 pathway regulation. In particular, several natural or nutraceutical products are suggested to have health-ameliorating effects or antiaging and anticancer effects. Such effects are modulated by PI3K/PTEN/AKT/mTORC1/GSK-3 signaling axis. Namely, three medicinal plant-derived substances are involved in the above-cited regulation: curcumin (CUR) *Curcuma longa*, berberine (BBR) Berberiscoptes, and resveratrol (RES), the latter especially present in red grapes. CUR acts by increasing the total level of GSK-3β in NCCIT human embryonic carcinoma cells with apoptosis induction, and a plethora of studies in the last years underlined the favorable impact of CUR on PI3K/PTEN/AKT/mTORC1/GSK-3 pathway in different types of cancer [139] and pathologies such as neurological diseases [140], obesity [141], diabetes [142], and cardiovascular disease [143]. BBR and RES act on PI3K/PTEN/AKT/mTORC1/GSK-3 pathway with beneficial effects on diabetes, cardiovascular diseases, neurological disorders, and cancer [144].

In conclusion, dietary or pharmacological administration of these compounds may represent, at least to some extent, potential alternatives to conventional drugs and still underlies the efficacy of GSK-3 modulation in counteracting aging-related pathologies.

9. Conclusions

Altogether, the studies summarized in the present review show that GSK-3 controls numerous cellular processes, plays an important role in autophagy regulation, and is involved in many human diseases. Further investigating substrate specificity and regulation of GSK-3 activity has important implications for potential therapeutic intervention.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Romina Mancinelli, Guido Carpino, Antonio Facchiano, Elio Ziparo, and Claudia Giampietri contributed equally to this work.

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